

Does HIV accelerate the aging process?

An assessment of clinical, ophthalmic and serum parameters in HIV-infected individuals in South Africa



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Declaration

I, Sophia Pathai, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature

Date: 11th January 2013



Abstract

HIV-infected individuals are at increased risk of age-related non-AIDS morbidity and mortality compared with HIV-uninfected persons. It is speculated that HIV-infected individuals may not only be aging chronologically, but also undergoing accelerated biological aging. This is supported by clinical reports of conditions classically associated with the normal aging process appearing at an earlier age in HIV-infected persons compared to age-matched controls.

Chronological age is an imprecise measure of biological aging due to inter-individual differences in rates of aging and therefore 'biomarkers of aging' may be used to assess biological age. The eye may be a uniquely useful site as a model of aging. It is easily accessible for examination and several components can be measured and assessed objectively e.g. lens density, retinal vascular calibre, corneal endothelial cell counts and the retinal nerve fibre layer thickness.

This case-control study of 504 adults recruited from one district in Cape Town, South Africa assessed whether HIV-infected individuals have more advanced ocular aging, systemic frailty and cellular senescence than an HIV-uninfected group of similar age. Accelerated biological aging was demonstrated in HIV-infected individuals compared to their uninfected counterparts. HIV infection was also associated with frailty. Ocular parameters provided evidence of greater aging within the HIV-infected group, particularly objective measurement of retinal vascular calibre and lens density. These data suggest that as well as increased biological aging at a cellular and systemic level, ocular aging occurs as part of the accelerated aging phenotype in HIV infection.

This study provides novel data about accelerated biological aging in sub-Saharan Africa and a platform for addressing future research questions relating to accelerated aging trajectories in HIV infection, the relative contributions of the infection and antiretroviral therapy, and whether biological age is dependent upon the duration of untreated disease or nadir CD4 count. As the HIV-infected population continues to age and expand, accelerated biological aging may have wide-ranging implications for the burden and management of HIV-related morbidity.

Preface

The thesis for this PhD uses the “research/review papers” format, recently introduced by the London School of Hygiene and Tropical Medicine. It therefore includes a number of papers which have either been published in, accepted by, or are formatted for submission to peer-reviewed journals. The chapters listed in italics in the Contents are in this research/review paper format and include publication details in a cover sheet, including acknowledgement of the contributions of other people. The other chapters of the thesis are composed of “linking material” which includes information/data not covered in the papers and helps to make the thesis a coherent body. The linking material was written by Sophia Pathai.

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Dedication

The work presented herein is dedicated to the memory of my father Adam Pathai (1946-2011) who gave me love and wings to soar high.

Contributors to the research presented in this thesis

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Colin Cook	Professor of Ophthalmology, UCT	Access to eye clinic for participant evaluation and advice with study design
Leris D'Costa	Study coordinator	Designing data collection materials and databases; assistance with fieldwork
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Helen A. Weiss	Reader in Epidemiology and International Health	PhD advisory committee and analytical guidance
Tien Y. Wong	Professor of Ophthalmology, SERI	Provision of IVAN software and guidance in use
Robin Wood	Professor and Director of Desmond Tutu HIV Centre, UCT	Advice on conducting research in South Africa and access to clinical cohorts

DTHC = Desmond Tutu HIV Centre; LSHTM = London School of Hygiene & Tropical Medicine; SERI = Singapore Eye Research Institute; UCT = University of Cape Town

Chapter I

HIV infection and accelerated aging



Study participants attending Groote Schuur Hospital for clinical evaluation

**Introductory linking material about aging,
immunosenescence and frailty in relation to
HIV infection**

I. Background

I.1 Aging, frailty and immunosenescence

Aging can be conceptualized as ‘a process of accumulation of deficits, taking place in different individuals in different ways, with a variety of rates for different organ systems, depending on the interplay of intrinsic and extrinsic factors’ [1,2]. A functional definition is the ‘time-dependent decline of functional capacity and stress resistance associated with increased risk of morbidity and mortality’ [3]. Aging ranges from ‘successful’ aging to ‘pathological’ aging depending on the functional reserves of different physiological systems and the consequent appearance of disease. Frailty may reflect an intermediate, but distinct state between these two extremes [2]. Frailty is a concept of decreased physiologic reserve that increases patient risk of morbidity and mortality [4]. A core feature of frailty is ‘increased vulnerability to stressors due to impairments in multiple systems that lead to decline in homeostatic reserve and resiliency’ [5]. It is apparent that many of the attributes of frailty also apply to ‘biological’ aging, as well as an inter-relation with chronic disease (Figure 1a). Systemic low-level inflammation is strongly associated with aging and chronic age-related diseases, and is also associated with frailty [6]. Low-grade inflammation is linked to alterations of the immune response in the elderly, termed immunosenescence (Section 1.2). At a cellular and molecular level, aging is manifest as cellular (or replicative) senescence. Old/senescent cells are characterized by an arrest of cell proliferation and an altered pattern of gene expression with telomere shortening (Section 1.5). Thus, aging is a complex phenomenon occurring at molecular, cellular, organ and physiological levels.

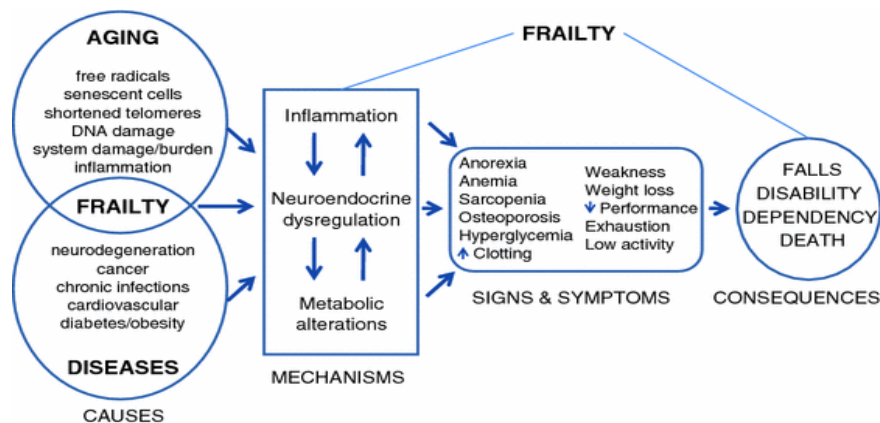


Figure 1a: Inter-relation between aging and disease and relationship to frailty, from Fulop et al [2]. Reproduced with permission from publisher.

1.1.1 Aging in treated HIV infection

Highly active antiretroviral therapy (HAART) has resulted in a reduction in mortality in HIV-infected people, largely due to prevention of AIDS-related events, but also due to a decrease in non-AIDS related events and deaths [7,8]. The age at diagnosis of HIV-infected individuals is increasing, as is the frequency of age-related conditions [9]. In the USA it is estimated that by 2015 more than 50% of the HIV-infected population will be over 50 years of age [10]. This trend is beginning to become apparent in some parts of sub-Saharan Africa where scale-up of effective HAART programmes and continued viral suppression are leading to a cohort of HIV-infected individuals who are in their fifties and sixties [11,12]. An emerging challenge is to understand the changing demography of the HIV-infected population, particularly in resource-constrained settings, and a shift from primarily treatment of acute opportunistic Infections (OIs) to the management of age-related conditions in the long term[13-15].

The Strategies for Management of Antiretroviral Therapy Study (SMART) group showed that HIV is associated with higher risk of several non-AIDS complications, and HAART reduces the risk of these events [16]. Although HAART can prevent AIDS and non-AIDS related morbidity and mortality, treatment does not fully restore health (Figure 1b). Patients receiving HAART are at increased risk of age-related non-AIDS-related morbidity and mortality compared with HIV-seronegative persons [17,18]. Several of these conditions are classically associated with the normal aging process but appear to occur at an earlier age in HIV-infected persons compared to age-matched controls. The emerging scenario is that of HIV population cohorts who are aging chronologically, but also likely to be undergoing accelerated physiological and immunological senescence.

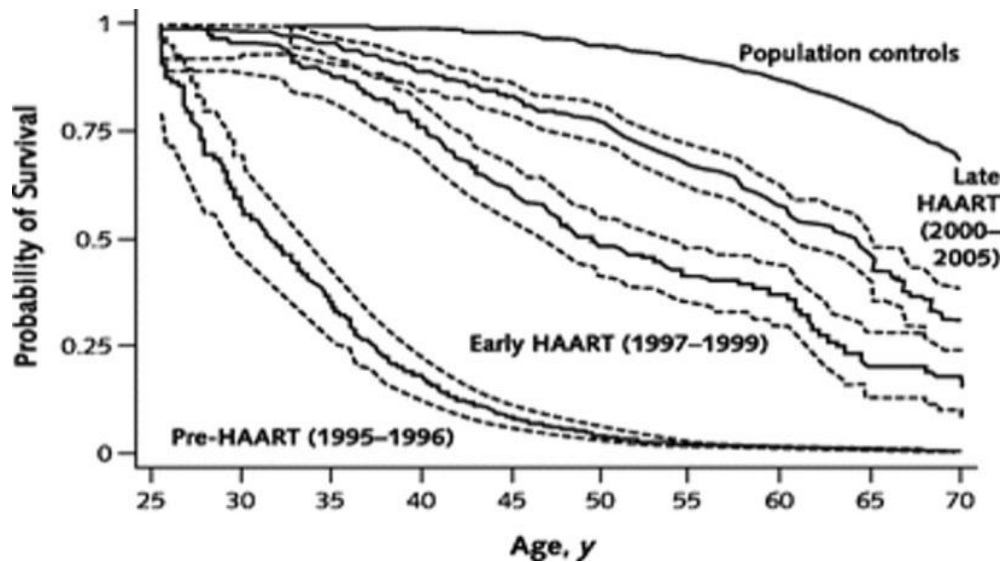


Figure 1b: From Lohse et al [18] Cumulative survival curve for HIV-infected persons (without hepatitis C co-infection) and persons from the general population. Reproduced with permission from publisher.

1.1.2 Which non-AIDS-related diseases are involved?

In the context of premature or accelerated aging and HIV, the conditions involved should already be known to be associated with aging in the general population, and the rate of these conditions should be greater among young-HIV-infected patients than uninfected patients of similar age [19]. Conditions where this phenomenon has been noted are discussed:

Cardiovascular disease: Patients with HIV infection are at increased risk of cardiovascular disease compared to age- and gender-matched uninfected counterparts [20,21]. Studies using administrative and clinical databases in the USA have compared rates of cardiovascular events (outcome definitions vary) between HIV-infected and uninfected groups. Age-adjusted hospital-admission rates for coronary heart disease (CHD) in HIV-infected versus HIV-uninfected populations were 6.5 versus 3.8 per 1000 person-years respectively, $p=0.003$ [22]. Review of the clinical registry for two tertiary hospitals in Massachusetts found higher proportions of diabetes, hypertension and dyslipidemia in HIV-infected patients compared to HIV-uninfected individuals. The mean myocardial infarction (MI) rates were 11.13 vs. 6.98 per 1000 person-years, respectively ($P<0.0001$). MI rates were higher in HIV-infected patients in all age groups [23]. However in all of these studies it was not

possible to adjust for all cardiovascular risk factors (BP, lipids, smoking, family history). These studies could have been subject to ascertainment bias as HIV-infected individuals may have had more hospital encounters providing the opportunity for CHD diagnosis compared to their HIV-uninfected counterparts. Data from over 28,000 patients in the Veterans Administration (VA) cohort showed a significantly increased risk of acute MI in HIV-infected patients versus non-HIV-infected patients, with an adjusted hazard ratio of 1.94 (95% CI, 1.58–2.37) [24]. The increased risk of cardiovascular disease is thought to be mediated via inflammatory and immunologic pathways, ultimately leading to vascular damage and dysfunction, however the contribution of HIV-related parameters is unclear: In the VA cohort [24], HIV viral load and current CD4 count were not associated with acute MI risk. In contrast, increased vascular aging (as measured by coronary artery calcium levels) was associated with current CD4 count in an Italian cohort of HIV-infected individuals [25]. Low nadir CD4 count, high current CD8 count and viral load >50 copies/mL were associated with an increased risk of MI in a French case-control study [26]. In a Dutch cohort, high-level viremia (400 copies/mL) in participants on HAART, was associated with cardiovascular disease [27]. Cardiovascular risk may also increase with some classes of HAART, particularly protease inhibitors [21,28,29]. Subclinical structural and functional abnormalities of the cardiac and vascular system have also been noted in HIV infection, with a higher than expected prevalence of echocardiographic abnormalities in the Study to Understand the Natural History of HIV/AIDS in the Era of Effective Therapy (SUN Study) cohort [30]. Early atherosclerosis is also thought to play a key role in the increased risk of cardiovascular disease in HIV infection, in particular endothelial dysfunction [31-33]. Host responses to cytomegalovirus (CMV) may be abnormal in HIV infection and associated with clinical disease (see section 1.2.1). Cytomegalovirus antibody titers are increased in HIV-infected women and associated with sub-clinical cardiovascular disease [34].

Renal disease: The incidence of end-stage renal disease (ESRD) in US Veterans was compared to a matched population of HIV-uninfected US veterans [35]. Among the white population there was no raised risk of ESRD for HIV-infected patients compared to HIV-uninfected patients. However, Afro-Caribbean individuals with HIV were as likely to develop ESRD as Afro-Caribbean individuals with diabetes and

almost twice as likely to develop ESRD as white individuals with diabetes. Further analysis of this cohort showed that compared with patients with only HIV or diabetes, patients with both diagnoses were at significantly increased risk of progressive chronic kidney disease (CKD) even after adjusting for traditional CKD risk factors [36]. Impaired renal function has also been found to identify HIV-infected patients at increased risk of cardiovascular events [37].

Bone disease: A meta-analysis of studies published between 1966 and 2005 investigating the prevalence of reduced bone mineral density (BMD) in HIV-infected patients found that 67% of patients had reduced BMD, with 15% of this group having osteoporosis. Risk of fracture is also increased in older HIV-infected men, but has not been fully established in younger HIV-infected men, and does not appear to be increased in younger HIV-infected women [38,39].

Pulmonary disease: Analysis of the Veterans Aging Cohort Study Virtual Cohort found infectious and non-infectious pulmonary diseases to be increased among HIV-infected compared to HIV-uninfected Veterans. The two most common incident pulmonary diseases were bacterial pneumonia and COPD; opportunistic diseases were less common [40]. Similar findings of increased respiratory symptoms and higher prevalence of COPD in HIV-infected individuals were noted in a case-control study from Italy [41]. HAART is also a risk factor for irreversible airway obstruction after adjustment for smoking [42]. Higher plasma HIV viral load is associated with increased risk of spirometry-defined obstructive lung disease [43]. HIV is thought to have an effect on lung function and damage such that the effect of HIV on lung age was approximated to smoking an additional 25 pack-years in a case-control study of HIV-infected and uninfected injecting drug users [44].

Cancer: In resource-limited settings, the prevalence of some AIDS-defining cancers (ADCs) such as cervical cancer, Kaposi's sarcoma and non-Hodgkin's lymphoma still remains high. However, increasing numbers of non-AIDS-defining cancers (NADCs) are now seen in resource-limited settings and well-resourced environments [45]. The most commonly reported NADCs regardless of setting include Hodgkin's lymphoma, lung, hepatocellular and breast cancer [46]. Premature aging would manifest not only as an overall elevated cancer risk but also as a downward shift in

the distribution of ages at cancer diagnosis. This is supported by studies of lung [47], liver [48], anal [49], and colorectal cancer [50] that have noted ages at diagnosis 10 to 20 years younger among HIV-infected individuals compared with the general population. However, differences in age distribution between the underlying HIV and general populations may account for these observations. Shiels *et al* [51] evaluated the ages at diagnosis of several types of non-AIDS-defining cancers in HIV and general populations using data from the U.S. HIV/AIDS Cancer Match Study, adjusting for differences in age and other demographic characteristics between these populations. The age at cancer diagnosis did not differ between HIV-infected persons and the general population for most types of cancer. However, ages at diagnosis of lung (median, 50 vs. 54 years) and anal cancer (42 vs. 45 years) were significantly younger in persons with AIDS than expected in the general population ($p < 0.001$), and the age at diagnosis of Hodgkin's lymphoma was significantly older (42 vs. 40 years; $p < 0.001$).

Frailty: is a concept of decreased physiologic reserve that increases patient risk of morbidity and mortality [4]. A 'frailty-related' phenotype has been defined in the aged population as at least three of the following characteristics: unintentional weight loss (10lbs in past year), self-reported exhaustion, weakness (grip strength), slow walking speed, and low physical activity [52]. Using this definition the frailty prevalence for 55-year-old men infected with HIV for ≤ 4 years (3.4%; 95% CI, 1.3–8.6) was similar to that of uninfected men ≤ 65 years old (3.4%; 95% CI, 1.5–7.6) [53,54]. A prevalence of 9% was reported from a clinic population in the United States (US) (mean age 42 years) [55]. The Women's Interagency HIV Study (a prospective cohort in five US cities) found a prevalence of 12% in HIV-infected women with clinical AIDS (median age 41 years)[56]. In the Multicenter AIDS Cohort Study (MACS) (a longitudinal study of men who have sex with men), a frailty prevalence of 5-14% depending on age and duration of HIV infection was reported from 1994-2005 data [57,58] and 8% in 2009-2010 among men aged 40-49 years [59]. In the SUN study (a US observational cohort of HIV-infected adults, median age 47 years), 5% of participants were frail [60]. The variation in estimates is likely to be attributable to differences in study design and clinical demographics of patients recruited (e.g. HAART status, degree of immunodeficiency). Current CD4 count is a

strong, independent predictor of frailty [56-59]. Central obesity and fat redistribution have also been suggested as part of the frailty-like phenotype in HIV-infected individuals [61]. HIV-infected individuals are also at greater risk of falls with middle-aged HIV-infected adults having a high fall risk [62]. Multiple comorbidities, medications and functional impairment were predictive of falls, however HIV-related parameters were not.

Neurological disease: HIV-infected individuals have an increased risk of cerebrovascular events (non-traumatic subarachnoid haemorrhage, intra-cerebral haemorrhage, cerebral infarction, unspecified stroke or transient ischemic attack), and this risk is associated with IDU, low CD4 count and exposure to abacavir [63]. Aging independently raises the risk of a range of neurocognitive disorders including memory impairment and mild-to-severe dementia. Neuro-degeneration associated with HIV infection combined with increased life expectancy can also accelerate neurocognitive decline and development of HIV-I-associated neurocognitive disorders (HAND) in up to 50% of HIV-I-infected individuals [64-66]. HAND includes asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and the most severe form, HIV-I-associated dementia (HAD). The current higher prevalence of the milder form of HAND coincides with the aging population of HIV-infected individuals, and so aging may be an important risk factor for HAND [65,66]. In sub-Saharan Africa, there is a growing recognition of HAND in patients in care and treatment (33% for ANI 12% for MND and 2% for HAD) [67]. The Veterans Aging Cohort Study (VACS) Index is a combined index of age, traditional HIV biomarkers (HIV-I plasma RNA and current CD4 count) and non-HIV biomarkers (i.e. indicators of renal and liver function, anaemia [haemoglobin], and Hepatitis C co-infection). Higher VACS Index scores are predictive of mortality and some types of morbidity [68,69]. Higher VACS Index scores are also associated with neurocognitive impairment (NCI). Among the VACS components, older age, lower hemoglobin and Hepatitis C co-infection were most strongly linked to HIV-associated NCI. Hemoglobin and Hepatitis C continue to be associated with NCI in models including AIDS status [70]. Frail HIV-infected men participating in the MACS cohort were also found to have a 2-fold risk of HAND after adjustment for socio-economic and HIV-related factors [71].

1.1.3 Contributing factors – why are patients on treatment still at risk?

There are likely to be several contributing factors why antiretroviral-treated patients remain at risk for premature morbidity and mortality compared with HIV-uninfected individuals. Differential risk exposures may play an important role - HIV-infected individuals may differ from a general population not only in being infected with HIV but also in terms of 'risk' behaviours (e.g. alcohol, substance abuse, smoking) and socioeconomic factors. In the US Veterans Aging Cohort, HIV-infected veterans were more likely to have co-morbid conditions such as kidney disease, liver disease, substance misuse and multi-morbidity compared to age- and sex- matched uninfected veterans [72]. The MACS/WIHS (Multicenter AIDS Cohort Study/ Women's Interagency HIV Study) found a higher prevalence of risk factors such as low levels of high-density lipoproteins, elevated levels of triglycerides and smoking in HIV-infected individuals compared to matched controls when assessing coronary heart disease risk [28]. The differential exposures to risk factors between HIV-infected and uninfected populations and residual confounding could result in an apparent increased risk of age-related outcomes. To account for such confounding factors in epidemiologic analyses requires very well-characterized patients [19].

The direct toxicities of HAART may also contribute to age-related complications. The Data Collection on Adverse Events of Anti-HIV Groups (D:A:D) studies showed that long-term exposure to protease inhibitor (PI) treatment was independently associated with the risk of cardiovascular disease (due to their tendency to cause dyslipidemia), and also with recent use of nucleoside reverse transcriptase inhibitors (abacavir and didanosine) [73,74]. In general, antiretroviral drug toxicity may contribute to the risk of age-related complications.

Overall, it appears that non-AIDS-related events are more common in HIV disease, even after adjusting for age, exposure to HAART and differential risk exposures. There may be an independent role of HIV infection in causing accelerated aging, mediated by elevated inflammation levels despite HAART, and chronic T-cell activation.

1.2 Immunosenescence and inflammation in HIV infection

1.2.1 Immunosenescence

Immunosenescence is impairment in immunity resulting from age-associated changes in function in a variety of cells, involving changes to both innate and adaptive immunity and an imbalance between the two arms [75]. The immune system in the elderly is characterized by progressive enrichment of terminally differentiated T cells (CD8⁺ effector cells), a reduction in T-cell renewal, reversed ratio of CD4⁺ to CD8⁺ cells, increased T-cell activation and increased levels of several inflammatory markers. This translates into a general decline of the immune system, leading to immunosenescence [76]. Several of the immunological changes that occur in HIV-1-infected individuals are similar to those associated with normal aging in the HIV-1-uninfected elderly [4] (Table 1a and Figure 1c).

Continuous stimulation of the immune system by HIV may lead to a progressive loss of the naïve and memory T-cell pools, resulting in an imbalance of T cell phenotypes. Chronic HIV infection leads to T cell differentiation with the expansion of antigen-experienced cells that lose the expression of immune marker CD28, with increased expression of CD57. CD28 is a co-stimulatory molecule on T cells; binding to its ligand CD80 on antigen presenting cells leads to interleukin-2 (IL-2) production, telomerase activation and 'survival signals' for other T cells (Figure 1c). Loss of CD28 on CD4⁺ cells also alters its capacity to drive B cell proliferation and antibody production. Loss of CD28 on T cells is a key predictor of immune aging [76]. HAART-treated patients (median age 56 years, <50 HIV-1 RNA copies/ml, median CD4 count 724 cells/mm³) were found to have frequencies of senescent CD8⁺ T cells (CD57⁺ CD28⁻ phenotype) similar to older HIV-negative individuals (median age 88 years) [77].

Clinically, T cell activation and senescence predict subclinical carotid artery disease in HIV-infected women. Compared with HIV-uninfected women, frequencies of CD4⁺CD38⁺HLA-DR⁺, CD8⁺CD38⁺HLA-DR⁺, and CD8⁺CD28⁻CD57⁺ T cells were higher among HIV-infected women, including those who achieved viral suppression

while receiving HAART. Among HIV-infected women, adjusted for age, antiretroviral medications, and viral load, higher frequencies of activated CD4⁺ and CD8⁺ T cells and immunosenescent CD8⁺ T cells were associated with increased prevalence of carotid artery lesions as determined by carotid ultrasound [78].

Chronic cytomegalovirus (CMV) infection is also implicated in accelerated aging in the healthy elderly population [79,80]. Elderly CMV seropositive adults have lower CD4/CD8 ratios, lower naïve T-cell numbers and higher frequencies of the CD8⁺ CD28⁻ phenotype compared to CMV seronegative adults [81]. Long-term successfully treated HIV-infected individuals (with undetectable plasma HIV RNA levels) have also been found to have high levels of CMV-specific effector cells, similar to that observed in the elderly, but occurring at much younger ages [82]. These levels were similar to that observed in the elderly, but occurred at much younger ages in the HIV-infected cohort (median age 45 years). The possible role of CMV in vascular aging has also been described (Section 1.1.2) with HIV-positive women with increased CMV IgG levels more likely to have carotid artery stiffness [34]. In a longitudinal study, CMV/HIV co-infection was associated with the risk of non-AIDS events/deaths independently of other prognostic factors [83]. CMV and HIV may influence immunological aging possibly in an additive manner, however it has also been suggested that CMV may be the ‘smoking gun’ in immunosenescence among persons co-infected with both pathogens, increasing the rate of immunological aging and the development of chronic diseases of inflammatory aetiology, especially among those undergoing HAART [84]. Thus, CMV or HIV may be co-drivers of immunosenescence, or it may be that CMV accentuates or accelerates this mechanism.

A unifying concept of the immunological changes that occur with physiological aging has been coined “*inflammaging*” by Franceschi and colleagues [6]. Characteristics include increased serum levels of pro-inflammatory cytokines (e.g. IL-6) and coagulation markers, a role for common viruses such as CMV in driving an ‘immune risk phenotype’, and also a genetic component which may explain why some individuals age faster than others. These changes are also consistent with the inflammatory and immunological processes reported in HIV, and add weight to the concept of accelerated aging in HIV.

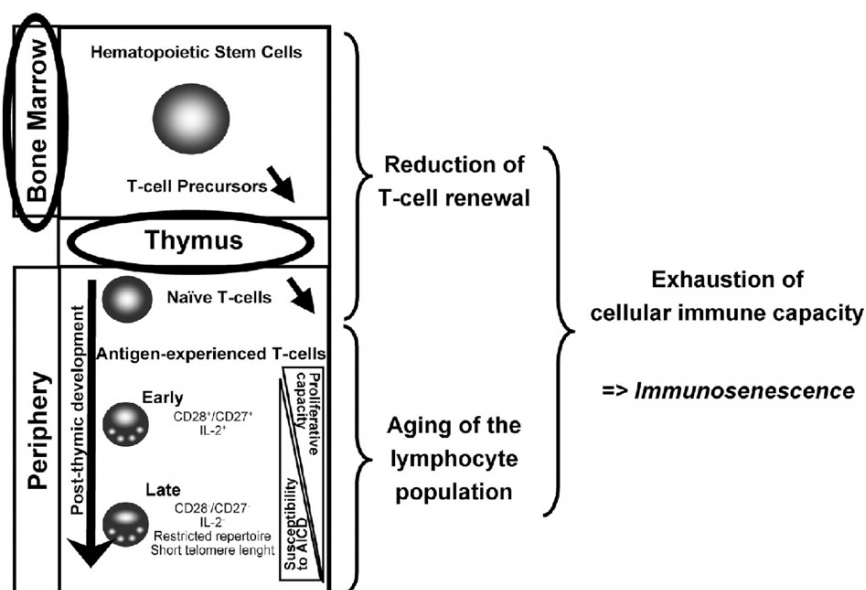
Whether HIV alone drives immunosenescence, acting as an accelerator of true biological aging, or as an independent causal factor for one or more co-morbid conditions remains to be established [19]. Measuring subclinical aging could be an important method to establish whether HIV is a true accelerant of the aging process.

Table 1a: Age and HIV-associated immune responses – adapted from Effros *et al.* [4]

Adaptive immune response	Change in HIV-infected patients, compared with age-matched controls	Change in aging persons, compared with young adults
B cells		
Naïve cell number	Normal to low	Normal to low
Memory cell number	Increased	Increased
Cytokine production	Increased	Normal
Primary responses	Decreased	Decreased
Memory responses	Low to normal	Normal
T cells		
Naïve cell number	Low	Low
Memory cell number	Low	Normal to high
Cytokine production	Low	Normal to high
CD28 expression	Very low	Very low
Senescent phenotype*	Very high	High

*Senescent phenotype consists of low CD28 expression, shortened telomeres, replicative incompetence and excessive production of inflammatory cytokines.
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Figure 1c: Proposed mechanisms of immunosenescence, from Appay *et al* [85].
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1.2.2 Inflammation

A potential mechanism to explain the increased risk of serious non-AIDS conditions could be via HIV-induced activation of inflammatory and coagulation pathways. The SMART study showed that several pro-inflammatory biomarkers (high-sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6) and D-dimer) were elevated in HIV-infected individuals, and remained so even with suppressed HIV RNA levels [86]. HIV virions (which may be non-infectious) continue to replicate and activate the innate and adaptive immune system to release pro-inflammatory mediators such as IL-6 that are associated with age-associated co-morbidities. Fibrinogen is an inflammatory biomarker in the clotting cascade. HIV-infected participants with fibrinogen levels in the highest tertile had a 2.6-fold higher adjusted odds of death than those with fibrinogen in the lowest tertile [87]. However, the study design means that causality cannot be definitively established; reverse causality may even be a factor where an already present disease process causes an increase in the biomarker level. In older people with the 'frailty phenotype', levels of IL-6, CRP and D-dimer are elevated [88]. Similarly, it is not known if elevated IL-6 or CRP causes this morbidity and mortality or if they are simply surrogate markers of a global inflammatory state.

1.2.3 Biomarkers of immune dysfunction in HIV

The similarities that HIV infection shares with aging in the general population, in terms of 'inflammaging' and immune senescence, is driven by chronic immune system activation. This has permitted the use of plasma and cellular biomarkers of immune system activation and dysfunction to predict outcomes, particularly opportunistic disease and mortality, in HIV-infected individuals [89].

Markers of inflammation (IL-6, hs-CRP), coagulopathy (protein S, protein C, and D-dimer) and gut microbial translocation (bacterial lipopolysaccharide [LPS] soluble CD14 [sCD14]) and cellular markers of immune dysfunction (CD38, CD57) are associated with chronic immune system activation and likely contribute to systemic inflammation, all of which may persist with HAART. Elevations of certain inflammatory or coagulopathic biomarkers, notably IL-6, hs-CRP, and D-dimer, have been independently associated with mortality and other clinical endpoints in large randomized clinical trials (Table 1b). As these biomarkers appear to add to the

predictive value of traditional tests used in management of HIV (e.g., CD4⁺ T-cell counts and HIV RNA), they have the potential to help guide treatment decisions [89].

Inflammatory markers are increasingly being evaluated in studies assessing their association with mortality and clinical endpoints in HIV infection – refer to Table 1b for further detail. Markers of immune dysfunction for further evaluation in clinical outcome studies have been presented in a review by Nixon and Landay [89] and in a report to the NIH Office of AIDS Research [90].

Indices and scores have also been developed using the biomarkers in tandem with clinical characteristics to provide practical and generalizable mortality risk index for HIV-infected individuals on ART [69]. The Veterans Aging Cohort Study (VACS) was used to develop the VACS Index, based on age, CD4 count, HIV-1 RNA, hemoglobin, AST, ALT, platelets, creatinine and Hepatitis C status. Among HIV-infected patients treated with ART, the VACS Index more accurately discriminates mortality risk than traditional HIV markers and age alone [91]. Addition of D-dimer and sCD14, but not IL-6, improves the predictive accuracy of the VACS Index for mortality [68]. VACS Index scores have also been shown to discriminate risk and provide accurate mortality estimates over 1-5 years of exposures to ART for diverse patient subgroups from North America [92]. Indices that take into account ocular parameters have not been developed to date.

Table 1b (Overleaf): Summary of studies assessing mainly inflammatory biomarkers and their association with mortality and clinical endpoints in HIV infection

First author, year, country	Biomarkers measured	Study design and population	Results	Overall conclusions
Ross, 2009 [93] USA	Common carotid artery and internal carotid artery IMT, TNF- α , hsCRP, IL-6, MLO, sVCAM-1	Case-control 73 HIV+, 21 HIV-	All biomarkers at higher level in HIV group	Hs-CRP positively correlated with carotid IMT in both groups. In the HIV+ group: sVCAM-1 positively correlated with all inflammatory cytokine levels. sVCAM-1, MLO, TNF-a all associated with internal carotid artery
Neuhaus, 2010 [86] USA	hs-CRP, IL-6, cystatin C	Individuals 45–76 years: 494 HIV+ individuals in the SMART study and 5386 uninfected participants in the MESA study. Individuals 33–44 years: hs-CRP and IL-6 levels compared in 287 HIV+ participants in the SMART study and 3231 participants in the CARDIA study.	hsCRP and IL-6 levels 55% (P=0.001) and 62% (P=0.001) higher among HIV+ group than CARDIA group. Compared with levels in MESA study participants, hsCRP, IL-6, D-dimer, and cystatin C levels were 50%, 152%, 94%, and 27% higher, respectively (P=0.001, for each), among HIV+ participants. HIV+ participants receiving HAART who had HIV RNA levels \leq 400 copies/mL had levels higher (by 21% to 60%) (P= 0.001) than those in the general population, for all biomarkers.	hsCRP, IL-6, D-dimer, and cystatin C levels are elevated in persons with HIV infection Remain so even after HIV RNA levels are suppressed with HAART

Kuller, 2008 [94] USA	hsCRP, IL-6, amyloid A, amyloid P, D-dimer, prothrombin fragment 1 β 2.	Case-control study as part of SMART 85 HIV+ deaths 170 HIV+ 'controls'	Higher levels of hsCRP, IL-6, and D-dimer at study entry significantly associated with an increased risk of all-cause mortality. Unadjusted ORs (highest versus lowest quartile) 2.0 (95% CI, 1.0–4.1; p= 0.05), 8.3 (95% CI, 3.3–20.8; p =0.0001), and 12.4 (95% CI, 4.2–37.0; p =0.0001), respectively.	IL-6 and D-dimer strongly related to all-cause mortality.
Sandler, 2011 [95] USA	I-FABP, LPS, sCD14, EndoCAb, and 16S (rDNA) measured in baseline plasma samples.	Nested case control from SMART study HIV+ group composed of: 74: died; 120: developed CVD; 81: developed AIDS Matched uninfected controls 2:1	Subjects with highest quartile of sCD14 levels had a 6-fold higher risk of death than did those in the lowest quartile (95% confidence interval, 2.2–16.1; P=0.001) No other marker was significantly associated with clinical outcomes. I-FABP, LPS, and sCD14 were increased and EndoCAb was decreased in study subjects, compared with healthy volunteers. sCD14 level correlated with levels of IL-6, C-reactive protein, serum amyloid A and D-dimer.	sCD14, is an independent predictor of mortality in HIV infection.

Ledwaba, 2012 [96], South Africa	Pre-ART plasma from patients with advanced HIV was used to measure hs-CRP, IL-6 and D-dimer	Nested case-control study 187 HIV+ deaths 359 HIV+ 'controls' matched on age, sex CD4 count	Median baseline biomarkers levels for cases and controls, respectively: 11.25 vs. 3.6 mg/L for hs-CRP; 1.41 vs. 0.98 mg/L for D-dimer; 9.02 vs. 4.20 pg/mL for IL-6 (all p=0.0001). Adjusted OR for the highest versus lowest quartile of baseline biomarker levels: 3.5 (95% CI: 1.9–6.7) for hs-CRP; 2.6 (95%CI 1.4–4.9) for D-dimer; 3.8 (95% CI: 1.8– 7.8) for IL-6. D-dimer and IL-6, but not hs-CRP, significantly lower at month 6 after commencing ART compared to baseline (p=0.0001)	Among patients with advanced HIV disease, elevated pre-ART levels of hs-CRP, IL-6 and D-dimer strongly associated with early mortality after commencing ART.
Gerena, 2012 [97] USA	Soluble and cell-associated IR levels, IRS-1 levels IRS-1 tyrosine phosphorylation Presence and severity of HAND	Retrospective cross-sectional study 34 HIV+ females 10 controls Hispanic-Latino Longitudinal Cohort of Women	HIV+ women had significantly increased levels of intact or full-length sIR in plasma (p=0.001) and CSF (p=0.005) relative to controls. Stratified by HAND, increased levels of full-length sIR in plasma were associated with the presence (p=0.001) and severity (p=0.005) of HAND. A significant decrease in IRS-1 tyrosine-phosphorylation in the WCP was also associated with the presence (p=0.02) and severity (p=0.02) of HAND.	IR secretion is increased in HIV+ women Increased IR secretion is associated with cognitive impairment in these women.

Armah, 2012 [98] USA	IL-6, D-dimer, and sCD14	1525 HIV+ 843 uninfected VACS participants	<p>Elevated IL-6 in HIV+ individuals with HIV-1 RNA \geq500 copies/mL or CD4 count $<$200 cells/μL (OR:1.54; 95%CI: 1.14–2.09; OR, 2.25; 95% CI, 1.60–3.16, respectively)</p> <p>Elevated D-dimer (OR, 1.97; 95% CI, 1.44–2.71, OR, 1.68; 95% CI, 1.22–2.32, respectively).</p> <p>Higher prevalence of elevated sCD14 in HIV-infected veterans with a CD4 cell count $<$200 cells/μL compared to uninfected veterans (OR, 2.60; 95% CI, 1.64–4.14).</p>	<p>Ongoing HIV replication and immune depletion significantly contribute to increased prevalence of elevated biomarkers of inflammation, altered coagulation, and monocyte activation.</p> <p>Associations persisted after restricting analysis to veterans without known confounding comorbid conditions.</p>
Keating, 2011 [99] USA	Multiplex assays of 32 cytokines	<p>Cross-sectional study - participants in the Women's Interagency HIV Study.</p> <p>HIV+ on HAART, n=17</p> <p>Non-controllers*, n=14</p> <p>Uninfected, n=17</p>	<p>Significant differences between non-controllers and uninfected participants for several markers:</p> <p>Elevated IP-10 and TNF-a</p> <p>Decreased IL-12(p40), IL-15, and FGF-2.</p> <p>Biomarker levels among HAART women more closely resembled the uninfected group, with the exception of TNF-a and FGF-2.</p> <p>Secondary analyses of combined HAART and non-controller groups: IP-10: positive correlation with viral load and negative correlation with CD4⁺ T-cell counts.</p> <p>VEGF, EGF, and FGF-2: positive correlation with increased CD4⁺ T-cell counts.</p>	<p>Untreated, progressive HIV infection associated with decreased serum levels of cytokines important in T-cell homeostasis (IL-15) and T-cell phenotype determination (IL-12 and increased levels of innate inflammatory mediators such as IP-10 and TNF-a.</p> <p>HAART associated with cytokine profiles that more closely resembled those of HIV-uninfected women.</p>

Kaplan, 2012 [100] USA	Six semi-annual measurements: soluble sCD14, TNF-a, soluble IL-2 receptor, IL-6, IL-10, monocyte chemoattractant protein 1, D-dimer, fibrinogen, and cIMT.	Women's Interagency HIV Study: 127 HIV+ women pre and post HAART 127 HIV-uninfected controls.	Relative to HIV-uninfected controls, HAART-naive HIV-infected women had elevated levels of sCD14, TNF-a, soluble IL-2 receptor, IL-10, monocyte chemo-attractant protein 1 and D-dimer (all P<0.01). Elevated biomarker levels declined after HAART. Although most biomarkers normalized to HIV-uninfected levels, in women on effective HAART, TNF-a levels remained elevated compared with HIV-uninfected women (+0.8 pg/mL, P = 0.0002). Higher post-HAART levels of soluble IL-2 receptor IL-6, and D-dimer associated with increased cIMT	Untreated HIV infection is associated with abnormal hemostasis (eg, D-dimer), proatherogenic (eg, TNF-a), and anti-atherogenic (eg, IL-10) inflammatory markers. HAART reduces most inflammatory mediators to HIV-uninfected levels. Increased inflammation and hemostasis are associated with subclinical atherosclerosis in recently treated women.
Mangili, 2012 [101] USA	cIMT and hs-CRP	Longitudinal study 327 HIV+ participants Median follow-up time 3.1 years	38 (11.6%) participants died since study enrolment. cIMT significantly higher in those who died and more likely to have cIMT above the 75th percentile Those who died had higher hsCRP than those alive and more had hsCRP values above 3 mg/L cIMT and hsCRP levels significantly associated with mortality (HR = 2.74, 95% CI 1.26–5.97, p = 0.01; HR = 2.38, 95% CI 1.15–4.9, p = 0.02).	Strong association of carotid IMT and hsCRP with all-cause death despite being similar with respect to exposure to HAART medications.

Margolick, 2012 [102] USA	FP+ or FP- at two consecutive study visits	MACS cohort – 602 men 117 HIV-/FP- 20 HIV-/FP+ 393 HIV+/FP- 72 HIV+/FP+	In HIV+ FP+ men higher concentrations of IL-6, TNF- α ; IL-8, IP-10, MCP-4, and TARC; and C-reactive protein Differences between FP+ and FP- HIV- men of similar magnitude but not significant CRP similar between HIV- FP+ and FP-.	Inflammatory markers had significant associations with FP in HIV+ men. Elevated IL-6, TNF- α , and CRP suggest monocyte activation. Elevation of IP-10 consistent with T-cell activation.
Kalayjian, 2010 [103] USA	sTNFR-1, sCD27, sCD40L, sTRAIL, HIV RNA level, CD4 cell count, IL-6, CD8 T cell activation, CD-38 and HLA.	Case control study Cases, n=41; Controls, n=111 HAART-naive in ACTG protocol 384 received either stavudine and didanosine/ lamivudine and zidovudine, in combination with either nelfinavir, efavirenz, or both. Follow-up every 4 weeks to 24 weeks. HAART-naive in ACTG protocol 5015 received a uniform regimen of stavudine and emtricitabine with ritonavir-boosted lopinavir. Follow-up every 8 weeks or every 12 weeks.	Higher levels of sTNFR-1 associated with older age, lower Hb level, lower CD4 cell count, lower eGFR, higher pVL, and higher levels of each of the other activation markers (except TRAIL and CD8 T cell activation).	New AIDS-defining illness or death was associated with: older age, female sex, lower baseline Hb, and higher baseline sTNFR-1, sCD27, sCD40L, and IL-6.

Abbreviations: (arranged alphabetically)

16S(rDNA), 16S ribosomal DNA; 95% CI, 95% Confidence Interval; ACGT, AIDS Clinical Trials Group; AIDS, Autoimmune deficiency syndrome; CARDIA, Coronary Artery Development in Young Adults; cIMT, carotid intima-media thickness; CSF, cerebrospinal fluid; HIV, Human immunodeficiency virus; CVD, Cardiovascular Disease; EGF, Epidermal Growth Factor; eGFR, estimated Glomerular Filtration Rate; EndoCAb, endotoxin core antibody; FGF-2, Fibroblast Growth Factor; FP, Frailty Phenotype; HAART, Highly Active Anti-Retroviral Treatment; HAND, HIV-1-associated neurocognitive disorders; Hb, Haemoglobin; HLA, human leukocyte antigen; hsCRP, high-sensitivity C-reactive protein; I-FABP, Intestinal fatty acid-binding proteins; IL-12, Interleukine-12; IL-15, Interleukine-15; IL-2, Interleukine-2; IL-6, Interleukine-6; IP-10, Interferon gamma-induced protein-10; IQR, Interquartile range; IR, Insulin Receptor; IRS-1, Insulin Receptor Substrate-1; LPS, lipopolysaccharide; MACS cohort, the Multicenter AIDS cohort study; MESA, Multi-Ethnic Study of Atherosclerosis; MLO, myeloperoxidase; OR, Odds Ratio; pVL, plasma viral load; RNA, Ribonucleic acid; sCD14, soluble CD14; sCD40L, soluble CD40 ligand; sIR, soluble IR; SMART, Strategies for Management of Antiretroviral Therapy Study; sTNFRs, soluble TNF superfamily receptors; sTRAIL, TNF-related apoptosis-inducing ligand; sVCAM-1, soluble vascular cell adhesion molecule-1; TNF-a, tumor necrosis factor-alpha; VACS, Veterans Aging Cohort Study; VEGF, Vascular endothelial growth factor; WCP, (CSF) white cell pellets.

* Antiretroviral therapy naïve and had a viral load more than 10000 RNA copies/ml for at least one of two time points separated by 6 months

1.3 Para-inflammation and aging

The immune response is thought to vary under different physiological conditions, and responds according to the type of ‘insult’. In basal conditions, the immune system maintains tissue homeostasis. Infection and tissue injury lead to full inflammation, whereas chronic tissue stress (noxious stress conditions such as hyperglycemia or hypercholesterolemia) initiates mild low-grade inflammation. Medzhitov has coined the term “*para-inflammation*” to describe this intermediate tissue adaptive response (Figure 1d) [104]. The outcome of para-inflammation is to maintain tissue homeostasis and monitor tissue malfunction. However, chronic para-inflammation can lead to disease progression due to changes in homeostatic set points. Several conditions are thought to have characteristics of para-inflammation; allergy and obesity may have maladaptive responses to noxious stressors due to shift of homeostatic set points. Type 2 diabetes, atherosclerosis and neurodegenerative disorders may occur as a result of dysregulation of para-inflammation [104]. Aging is one of the main factors that may lead to increased tissue stress, and an imbalance in tissue homeostasis that may ultimately lead to pathology. The magnitude of a para-inflammatory response may also increase and produce age-related diseases.

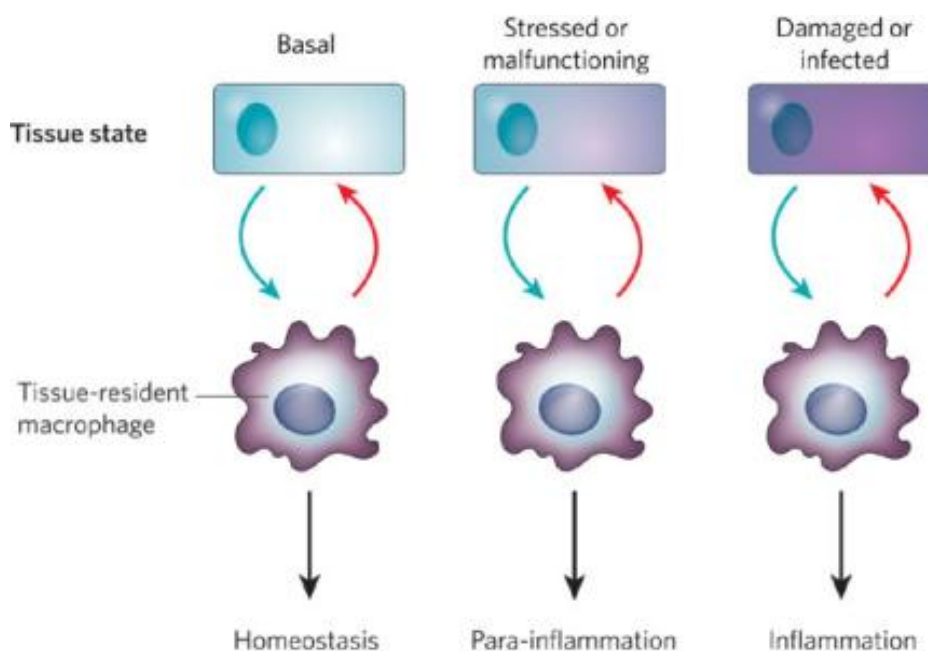


Figure 1d: Concept of para-inflammation - from Medzhitov [104]. Reproduced with permission from publisher.

1.4 Para-inflammation and retinal aging

Xu et al [105] suggest that para-inflammation is an important process within the aging retina. The retina is a highly differentiated neuro-ectodermal tissue. The neuroretina and retinal pigment epithelium (RPE) form a functional unit of the visual system (Figure 1e). In the retina, a number of conditions are age-related, including primary open angle glaucoma, diabetic retinopathy and age-related macular degeneration. The retina has an endogenous immune system that is coordinated by immune cells such as microglia, dendritic cells and perivascular macrophages in tandem with the immunological functions of the RPE [105,106]. Together, these cells are involved in maintaining retinal homeostasis in the presence of stressors (e.g. tissue injury). Retinal aging is accompanied by activation of gene sets either modifying immune responses or tissue stress/injury responses, which are involved in local inflammatory responses [107]. In the aging retina, two inflammatory pathways are affected as a result of age-related tissue stress: the complement cascade and retinal microglia activation [108]. Excessive uncontrolled complement or microglial activation may produce overt inflammatory responses with marked release of cytokines/chemokines causing irreparable damage to the neuroretina [105]. Immunological changes that occur in HIV infection could lead to excessive uncontrolled complement or microglial activation within the retina. This in turn could cause low-grade tissue stress and para-inflammation leading to changes in the retina normally seen in physiological aging.

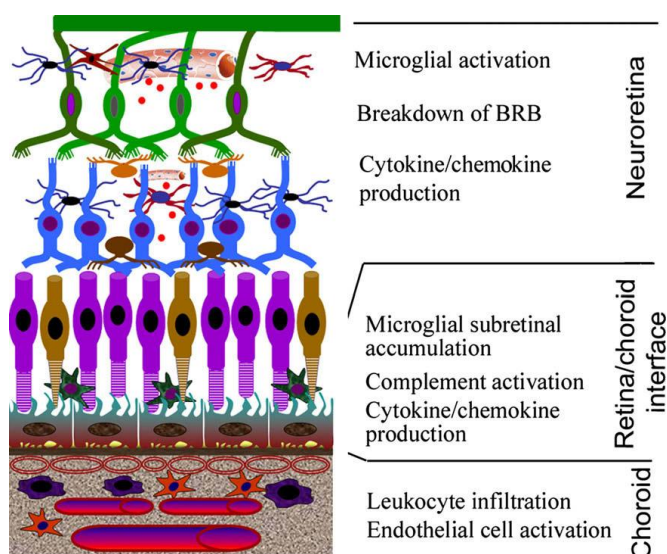


Figure 1e: From Xu et al [105] – Histology of the retina and para-inflammatory processes. Reproduced with permission from publisher.

1.5 Biomarkers of aging

Given the potential differences in lifespan, rate of aging, and differences in the same parameters between individuals, the chronological passage of time may not represent the best measure of rate of aging. The disconnect between chronological age and lifespan has led to the search for biomarkers of aging (BoA). The term biomarker of aging has been defined as a “biological parameter of an organism that either alone or in some multivariate composite will better predict functional capability at some late age, than will chronological age” [109]. It may be difficult to separate biomarkers of disease from BoA, as aging could reflect the sum of a lifetime of disease processes [110].

1.5.1 Telomeres

Cellular senescence is characterized by critical telomere shortening. Telomeres are repeating sequences of DNA at the extreme ends of eukaryotic chromosomes. They have roles in chromosomal protection, nuclear attachment and replication. They shorten with cell division due to the inability of the DNA-copying mechanism (DNA polymerase) to synthesize the very end of the DNA sequence. When telomeres reach a critically short length, a DNA damage signal is transmitted leading to irreversible cell cycle arrest or replicative senescence [111]. Progressive shortening of lymphocyte telomeres is characteristic of immune senescence, and may affect lymphocyte function [112].

Telomeres in man shorten with increasing chronological age [113] and telomere attrition has been noted in an increasing number of diverse pathologies e.g. cardiovascular disease, hypertension [114-116], renal transplant dysfunction [117], and psychological disorders [118]. Telomere erosion has also been associated with high mortality in kidney disease and heart disease [119,120]. A U-shaped association has been noted for telomere length in colorectal cancer in women, with both very short and very long telomeres associated with increased risk of colorectal cancer [121]. In healthy subjects, shortened lymphocytic telomeres are associated with increased mortality, particularly after infection [122]. Interpretation of epidemiological studies using telomere length can be difficult, as short telomeres could be both a cause and consequence of biological aging [123]. Given the variability of telomere length between individuals of the same chronological age and between

different tissues, and that there are no "normal" values, large sample sizes are required as well as inclusion of suitable controls [124]. Longitudinal studies, including birth cohort studies, would be the preferred study design. Telomere length does not satisfy the strict criteria for a BoA, but does add predictive power to that of chronological age [125].

Cytotoxic CD8 T-cells that lose expression of CD28 (as in aging) have shorter telomere lengths compared to any other T and B cell subsets [126]. Chronic HIV infection appears to exacerbate the situation: CD28- CD8+ T-cells from HIV-infected patients had significantly shorter telomere lengths compared to CD28- CD4+ T-cells of uninfected controls. The telomere lengths in the HIV-infected group were similar to those found in elderly non-infected controls [127].

Telomere attrition is affected by psychosocial confounders, genetics and potentially by nucleoside reverse transcriptase inhibitors (NRTIs) [128], particularly as human telomerase comprises a reverse transcriptase sharing homology with the HIV reverse transcriptase [129]. It is plausible that ART may inhibit its activity, leading to differences in telomere lengths between ART-naïve and treated groups. Previous studies have produced inconsistent findings regarding such an association [130,131]. *In utero* or childhood exposure to NRTIs does not affect leukocyte telomere length (LTL), and uncontrolled viremia rather than duration of ART exposure may be associated with acceleration of blood telomere attrition [132,133]. There are few data to date evaluating biological aging in HIV-infected individuals compared to age- and gender-matched controls. A case-control in Canada found that beyond advancing age, HIV+ status, active HCV infection and low income were the strongest predictors of LTL with no evidence that ART-related factors impact LTL [134].

1.5.2 Other biomarkers of aging

Measurement of the cell cycle regulator CDKN2A also provides a method of assessing bio-aging [124]. CDKN2A functions to hold a cell in a state of growth arrest. Levels of CDKN2A can be measured by reverse transcriptase polymerase chain reaction and generating a standard assay is feasible. Increasing levels of CDKN2A transcriptional expression occur with increasing age and in solid organs

and peripheral blood leucocytes [135-137]. Levels of CDKN2A also increase with decreasing organ function, and levels positively associate with levels of IL-6 [136].

A major marker of oxidative damage of nucleic acids is the 8-hydroxy-2'-deoxyguanosine (8-OHdG) adduct. It is formed when reactive oxygen species (ROS), especially the hydroxyl radical (OH•) act on deoxyguanine in DNA [138]. 8-OHdG can alter gene expression and levels of 8-OHdG are inversely related to life span in mammals and increase especially in mitochondrial DNA with age [3,139]. 8-OHdG is also elevated in degenerative diseases such as Parkinson's disease, diabetes, cystic fibrosis and muscular dystrophy [140]. Inflammation accelerates telomere attrition [111] and prolonged oxidative damage inhibits telomerase activity and accelerates telomere shortening; this effect may be mediated by the formation of 8-OH-dG [141].

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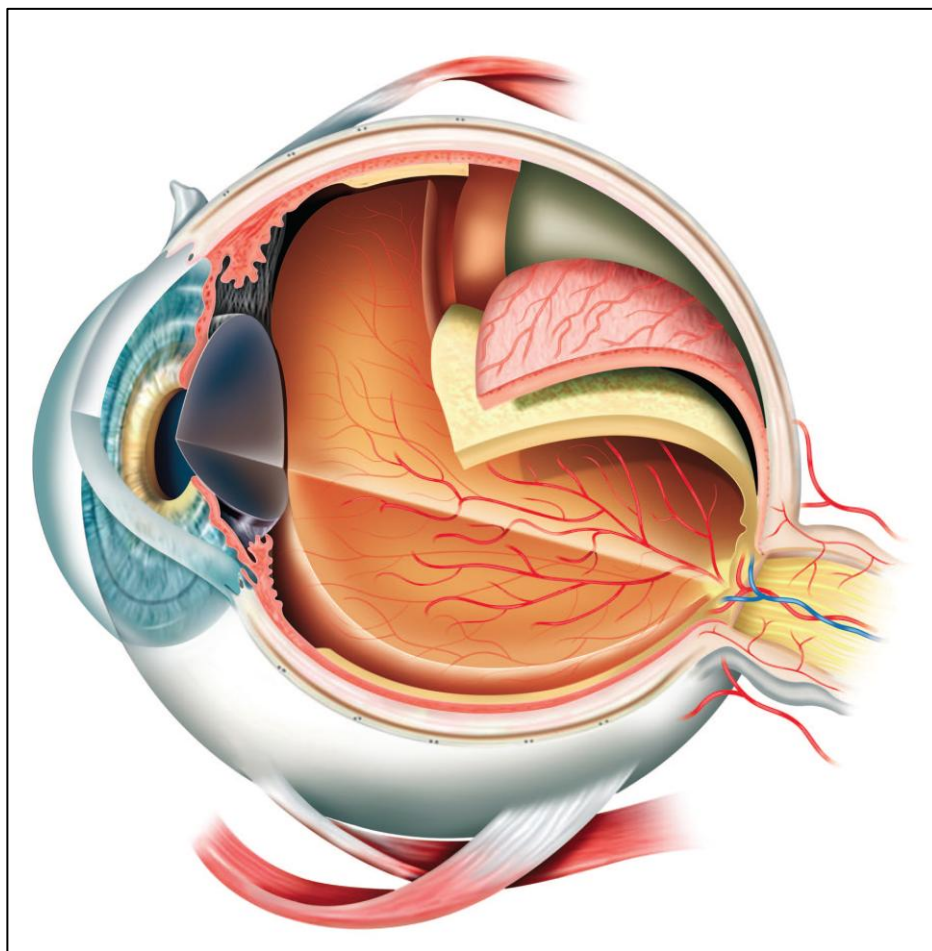
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Chapter 2

The Eye as a Model of Ageing in Translational Research:

Molecular, Epigenetic and Clinical Aspects



Schematic of the structural and functional components of the eye

Review paper containing detailed summaries about the use of the eye in translational research and as a model of aging

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? ***Ageing Research Reviews***
 - 1.2. When was the work published? ***December 2012***
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion ***N/A***


 - 1.3. Was the work subject to academic peer review? ***Yes***
 - 1.4. Have you retained the copyright for the work? ***No***

If yes, attach evidence of retention

If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work ***See Appendix 7***
2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? ***N/A***
 - 2.2. List the paper's authors in the intended authorship order
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The Eye as a Model of Ageing in Translational Research – Molecular, Epigenetic and Clinical Aspects

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Abstract:

The eye and visual system are valuable in many areas of translational research such as stem cell therapy, transplantation research and gene therapy. Changes in many ocular tissues can be measured directly, easily and objectively *in vivo* (e.g. lens transparency; retinal blood vessel calibre; corneal endothelial cell counts) and so the eye may also be a uniquely useful site as a model of ageing. This review details cellular, molecular and epigenetic mechanisms related to ageing within the eye, and describes ocular parameters that can be directly measured clinically and which might be of value in ageing research as the translational “window to the rest of the body”. The eye is likely to provide a valuable model for validating biomarkers of ageing at molecular, epigenetic, cellular and clinical levels. A research agenda to definitively establish the relationship between biomarkers of ageing and ocular parameters is proposed.

Keywords:

Biomarker; eye; ophthalmology; epigenetics; translational research

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MAIN TEXT

1. Introduction

Ophthalmology is increasingly recognized as being at the forefront of translational research (Stahl and Smith, 2010). The unique access to and visibility of ocular tissues and range of visual functions permits investigation of a wide variety of physiological and pathological mechanisms. Stem cell biology, transplantation and gene therapy are all areas of research where the eye plays a prominent role in animal models as well as in clinical research. In this review we make the case that the eye can also play a key role in ageing research, with many ocular age-related changes having translational applications to other body systems. Further support for this argument comes from studies which show that visual impairment is a significant predictor of mortality (Lott et al., 2010). Cataract, glaucoma and age-related macular degeneration (AMD) are important causes of low vision in the elderly. Moreover, poor visual function is related to frailty (Klein et al., 2003; Kulmala et al., 2011), which is an age-related clinical concept of increased vulnerability to stressors due to impairment of multiple systems, resulting in increased risk of morbidity and mortality (Bergman et al., 2007; Fried et al., 2001; Fulop et al., 2010). AMD and low vision are also related to depression and increased morbidity (Brody et al., 2001; Huang et al., 2010; Mathew et al., 2011; Popescu et al., 2012)

Evolution, and behavioural studies of humans and animals, demonstrate the vital importance of vision. Studies of infants who are blind from birth show that vision is vital for early sensory-motor and psychosocial development, as it is the sense that co-ordinates the other sensory inputs. However, light energy is highly damaging to tissues, and the human eye has developed a range of mechanisms that protect its unique structures and functions from oxidative stress and other damaging insults. Failure of these mechanisms can lead to pathology and presentation of age-related ocular conditions such as cataract, primary open angle glaucoma and age-related macular degeneration (AMD). Virtually all tissues of the eye are affected by ageing; the most well known pathologies are age-related development of opacification of the lens (cataract) and age-related macular degeneration. Indeed, epidemiological research has demonstrated that individuals with cataracts have a significantly higher mortality rate than those without, even after adjusting for known confounders. This suggests that biochemical mechanisms in ocular lens may reflect ageing processes elsewhere in the body (Hennis et al., 2001; Truscott, 2005; Truscott and Zhu, 2010; West et al., 2000). The retinal ganglion cells in the eye can also provide information relating to neuronal ganglion function in ageing and age-related disease such as Alzheimer's disease and glaucoma (Curcio and Drucker, 1993; Nag and Wadhwa, 2012; Osborne, 2008). Further examples of the age-related changes within the eye include loss of corneal endothelial cells, changes in the calibre of the retinal vasculature, loss of visual function (e.g. visual acuity; contrast sensitivity) and thinning of the retinal tissue layers, all of which can be measured easily, quickly, objectively and non-invasively. Many of these ocular age-related changes also have systemic associations or correlates with ageing in other end-organs or body systems, and these changes may be

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easier or less invasive to measure in the eye than other organs or systems (*Table 1*).

We present a comprehensive overview that proposes that the eye represents an ideal model for studying age-related processes at biological, clinical, epigenetic and molecular levels. The structural organization of the review leads the reader through (i) the rationale for the eye to be considered a model of ageing (ii) cellular and molecular mechanisms of ageing in the eye, (iii) epigenetic mechanisms and their role in age-related eye disease and (iv) ocular parameters that can be readily measured which might be of value in ageing research, using the eye as the translational “window to the rest of the body”. We conclude by discussing how the eye might represent a powerful new model for validating potential biomarkers of ageing and propose a research agenda to establish whether the eye is a suitable model.

2. Global ageing demographics and the need for biomarkers of ageing

Population ageing is rapidly changing: by 2017, for the first time in history, the number of people globally aged 65 years and older will outnumber children younger than 5 years (World Health Organisation, 2011). In addition there are substantial differences in the health and functional status of older populations in many developing countries as well as marked differences in health status within the UK and other developed countries (Lloyd-Sherlock et al., 2012). These complex variations in health and functional status are not fully understood, highlighting the need for translational age-related research within a global context. Basic science ageing research often employs survival curves and maximum life span as key end points for studies of effects of interventions, rather than healthspan or function. However disability, frailty and age-related disease onset are critical end points that need to be addressed in older populations (Kirkland and Peterson, 2009). Many age-related systemic diseases have ophthalmic manifestations including diabetes, hypertension, cerebrovascular disease, rheumatoid arthritis and systemic lupus erythematosus (SLE). Furthermore, there is increasing evidence that HIV infection may lead to premature ageing in infected individuals even with viral suppression and long-term anti-retroviral treatment (Deeks, 2009; Deeks and Phillips, 2009; Lohse et al., 2007; Onen et al., 2010). As the research agenda for ageing expands to address these new concepts and hypotheses it is vital to have easily measurable, objective and validated parameters or biomarkers of ageing that can be translated to other body systems.

Given the potential differences in lifespan, the concept of healthspan, rate of ageing, and differences in the same parameters between individuals, the chronological passage of time may not represent the best measure of ageing of biological systems. The disconnection between chronological age and lifespan has led to the search for biomarkers of ageing (BoA). The term biomarker of ageing has been defined as a “biological parameter of an organism that either alone or in some multivariate composite will better predict functional capability at some late age, than will chronological age” (Sprott, 2010). A

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research agenda for developing ageing research in the UK acknowledges that many lifestyle- and age-related chronic diseases share common pathways of early dysregulation (e.g. cardiovascular disease, Alzheimer's disease), and that the development of markers, diagnostic techniques and interventions that can be applied to prevent late stage disease is fundamental (Franco et al., 2007). The need for research into how to achieve healthy ageing in the context of life-time trajectories has also led to concept of the 'Healthy Ageing Phenotype' defined as " the condition of being alive, while having highly preserved functioning metabolic, hormonal and neuro-endocrine control systems at the organ, tissue and molecular levels" (Franco et al., 2009). With demonstrable molecular, epigenetic and clinical correlates of ageing, the eye may be a model system for validating potential biomarkers.

2.1 Criteria for a biomarker of ageing

Baker and Sprott (1988) have suggested the following criteria satisfy the requirement for a parameter to be considered a biomarker of ageing:

1. The rate of change of a biomarker must, at least in mathematical terms, reflect some measurable parameter which can be predicted at a later chronological age.
2. The biomarker should reflect some basic biological process of ageing and certainly not the predisposition toward a disease state or some error in metabolism.
3. The biomarker should have high reproducibility in cross species comparisons of functional or physiological age versus chronological age, particularly within the same classes and certainly within the same families of species.
4. Biomarkers should change independently with the passage of time and reflect physiologic (functional) age.
5. Assessment of biomarkers should be nonlethal in animal systems and should cause minimal trauma in humans.
6. The biomarker should be reproducible and measurable during a relatively short time interval compared to the life span of the animal.

3 Cellular and molecular mechanisms of ageing in the eye:

3.1 Para-inflammation and retinal ageing

Xu et al., (2009) have suggested that para-inflammation is an important process within the ageing retina. The retina is a highly differentiated neuroectodermal tissue. The neuroretina and retinal pigment epithelium (RPE) form a functional unit of the visual system. The retina has an endogenous immune system that is coordinated by immune cells such as microglia, dendritic cells and perivascular macrophages in tandem with the immunological functions of the RPE (Xu, 2009). Together, these cells are involved in maintaining retinal homeostasis in the presence of stressors (e.g. tissue injury). Retinal ageing is accompanied by activation of gene sets either modifying immune responses or tissue stress/injury responses (Chen et al., 2010). In the ageing retina, two inflammatory pathways are

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affected as a result of age-related tissue stress: the complement cascade and retinal microglia activation (Chen et al., 2010). Excessive uncontrolled complement or microglial activation may produce overt inflammatory responses with marked release of cytokines/chemokines causing irreparable damage to the neuroretina (Xu et al., 2009). This modified form of low-grade chronic inflammation (para-inflammation) may be initiated by several stressors, including oxidative stress or advanced glycation end products (see sections 5 and 6). Dysregulation of the complement pathway is also implicated in AMD; altered levels of both intrinsic complement proteins and activated products have been found in the circulation of patients with AMD. Complement activation may be triggered by oxidative stress, resulting from retinal exposure to incoming light (Khandhadia et al., 2012). This is, in part modified by genetic mechanisms: variation of the complement factor H gene (*CFH*) is associated with AMD (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005), with the Y402H variant conferring a 2-fold higher risk of late-AMD per copy in individuals of European descent (Sofat et al., 2012)

3.2 The retina as a model in neurodegenerative disease

The retina is derived from the neural tube, a precursor of the central nervous system (CNS). Because of their common origin and anatomical features, AMD in the retina and Alzheimer's Disease (AD) are thought to have many parallel characteristics (Ohno-Matsui, 2011). An important characteristic common to both is the presence of amyloid β ($A\beta$) in the senile plaques of the AD brain and in the drusen of AMD patients. Extracellular deposits are a common pathological hallmark of both AMD and AD. In AMD, the deposits are called drusen, and in AD, the deposits in the brain are referred to as senile plaques (Ohno-Matsui, 2011). The deposits share similar biochemical characteristics and contain amyloid forms with the presence of a wide spectrum of amyloid structures in drusen (Isas et al., 2010). Amyloid deposition may trigger chronic inflammation including complement activation in both disorders. Although $A\beta$ is deposited in the brain and in drusen, the phenotype is different between AD and AMD, and the genetic epidemiology of the two conditions is different. In sporadic or late-onset AD, the apolipoprotein-E (APOE) $\epsilon 4$ allele increases the risk of developing the disease in all ethnic groups (Farrer et al., 1997). Three additional genes, the complement receptor 1 (*CR1*), clusterin (*CLU*), and phosphatidylinositol-binding clathrin assembly protein (*PICALM*) are also novel susceptibility loci for late-onset AD in European ancestry populations (Jun et al., 2010). In contrast, variants of the complement factor H gene are associated with AMD (see Section 3.1).

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Microglia are the resident immune cells of the CNS having vital immuno-surveillance and tissue repair functions. One of the major proposed mechanisms for the pathological changes in the brains of AD patients is the activation of microglial cells. In a retinal microglia model of neurodegenerative disease, dynamic microglial responses to injury were found to be age dependent (Damani et al., 2011), and

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senescent alterations in their morphological and behavioural phenotypes may contribute to the pathogenesis of age-related neurodegenerative disease. The retina is thought to be an ideal model of the cellular and molecular characteristics of neurodegenerative diseases. This may be manifest functionally as loss of retinal ganglion cells and may reflect degenerative changes in the brain in these conditions. Indeed loss of retinal ganglion cells is implicated in the pathogenesis of glaucoma (Osborne, 2008), and CNS damage has been associated with glaucoma (Nucci et al., 2012), including atrophy of the lateral geniculate nucleus (Gupta et al., 2009). Retinal nerve fibre layer thinning in particular may act as a biological marker of neurological disease (*see section 8.7*).

3.3 Ocular basement membranes as a model for other basement membranes

Visualisation of the ultrastructure of the retina by electron microscopy has provided significant insight in to how basement membranes alter with increasing chronological age. The inner limiting membrane (ILM) is a basement membrane located at the border of the vitreal surface of the retina and the vitreous body. Human ILM undergoes age-dependent alterations including a dramatic increase in thickness, loss of the typical basement membrane ultrastructure and an increase in stiffness (Candiello et al., 2010). Physiologically this may lead to a lower rate of filtration and diffusion and a loss of vascular elasticity. Recently, a nanometer scale topographic and biomechanical profile of human ILM has been created under physiological conditions using Atomic force microscopy (ATF; a very high-resolution type of scanning probe microscopy) (Henrich et al., 2012). The ILM has an ultrastructure and a protein composition typical of most other basement membranes and, thus, provides a suitable model for determining their biophysical properties. Biological characteristics of the ILM from human eyes may provide information about how other basement membranes from other tissues undergo age-related changes (Candiello et al., 2007). Bruch's membrane (BM) is a pentalaminar structure, located between the retinal pigment epithelium (RPE) and the fenestrated choroidal capillaries of the eye. BM is an elastin- and collagen-rich extracellular matrix that acts as a 'molecular sieve' (Booij et al., 2010). The changes occurring in BM with age include increased cross-linkage of collagen fibres and increased turnover of glycosaminoglycans. In addition, advanced glycation end products (AGEs) (section 5) and fat accumulate in BM. These age-related changes may influence the normal age-related health of photoreceptor cells within the retina, but also the onset and progression of diseases like retinitis pigmentosa (RP) and AMD with formation of drusen deposits (Okubo et al., 1999). Within the cornea, Bowman's layer provides support to the anterior epithelial layer and is an acellular interface between the corneal epithelium and stroma. It may be visualised using spectral domain optical coherence tomography (SD-OCT) (Tao et al., 2011; Vajzovic et al., 2011). Similarly, Descemet's membrane in the cornea can also be visualised using *in vivo* confocal microscopy where changes with age have been noted (Jun et al., 2006).

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Section 3.4 The vitreous and ageing

The vitreous is a highly hydrated, almost acellular structure comprising 0.1% macromolecules, of which collagens are the most important building blocks (Ponsioen et al., 2010). Morphological age-related changes in the vitreous include a progressive increase in fluid-filled areas (synchysis), and an increase in optically dense structures (syneresis). These changes are less dependent on stressors, reactive oxygen species and disease, and thus represent physiological ageing changes. The age-related changes in the vitreous may also affect local structures; for example, low pO₂ is maintained at the posterior surface of the lens by the physical and physiological properties of the vitreous body, the gel filling the space between the lens and the retina. Destruction or degeneration of the vitreous body increases exposure of the lens to oxygen from the retina. Oxygen reaches the lens nucleus, increasing protein oxidation and aggregation and may lead to cataract formation (Beebe et al., 2011).

4. The lens as a model for macromolecular ageing

4.1 Long-lived proteins in the eye

There are several sites in the body where proteins are 'long-lived', including the eye, lungs and brain. These proteins may be subject to modification, either due to the intrinsic instability of some amino acid residues in proteins, or due to covalent modification by biochemicals produced during cellular metabolism (Hipkiss, 2006; Truscott, 2010). Truscott proposes that progressive damage to these long-lived proteins may contribute to the age-related decline in function, and that cumulative denaturation of proteins ultimately limits human life span (Truscott, 2010). In this way both presbyopia (age-related reduction in near vision; section 8.6) and cataract can be thought of as being direct outcomes of denaturation of lifelong proteins in the lens. For example, diminished function over time is related to protein damage, rather than DNA damage in the lens (McGinty and Truscott, 2006). Ageing may be partly attributable to loss of function due to protein damage rather than direct DNA damage per se, however it is equally possible that it is cumulative damage to genetic material (through loss of physiological repair processes) and thus consequential protein dysfunction and loss of function that leads to phenotypic ageing.

The lens is thought to be an ideal tissue for studying 'macromolecular ageing' in humans (Truscott and Zhu, 2010). It is also recognised as an excellent model of physiological and 'pathological' ageing (Michael and Bron, 2011). The lens is one of the few sites in the body which is also an avascular and immune privileged site (Wormstone and Wride, 2011). Thus, it is possible to explore roles for specific molecules in cell proliferation, differentiation and development because cells remain in place for life, and go through extreme differentiation, including removal of nuclei and cessation of protein synthesis. In addition, the centre of the lens is an enzyme free zone with a lack of active metabolism. This means the impact of thermal and chemical denaturation of proteins can be studied without confounding factors such as enzyme modifications. Lens nuclear proteins could be ideal to discover which reactive

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metabolites in the body are quantitatively of most importance for post-translational modifications of long-lived proteins (Zhu et al., 2010). This may be useful to understand if and how accumulation of altered polypeptides in the heart, lung and brain directly affects lifespan (Truscott, 2011). Human lenses contain crystallins; these proteins are soluble, compared to collagens and elastin elsewhere in the body that are insoluble.

4.2 Telomere length in lens epithelial cells

Telomeres are nucleoprotein complexes at chromosome ends, consisting of (TTAGGG)_n direct repeats bound to a range of proteins involved in maintaining cellular stability and viability (Shiels et al., 2011). They shorten with every cell division in response to intrinsic molecular factors and DNA damage, and short telomeres trigger a DNA damage response leading to cell death or senescence (von Zglinicki, 2012). Increased oxidative stress accelerates telomere attrition in somatic cells, resulting in premature cellular senescence and reduced organ function (Carrero et al., 2008) (see Section 6). Studies of human lens epithelial cells suggest that cataract formation is characterised by senescence of lens cells, accelerated by oxidative stress-induced DNA damage, modulation of the antioxidative capabilities by the normal and cataractous lens, and marked telomere shortening (Babizhayev et al., 2011; Babizhayev and Yegorov, 2010).

4.3 Cataract as a 'bio-indicator' for severe systemic disorders – role of the crystallins

Crystallins represent the major structural proteins of the eye lens - they make up 90% of its total dry mass and are responsible for the refractive power of the lens (Horwitz, 2000). The crystallins have been classified into α , β and γ -crystallins, reflecting their molecular weight. Besides their refractive roles, crystallins are also involved in several cellular pathways involving the stress response, apoptosis and cell survival at a systemic and ocular level (Andley, 2008). In particular, the α -crystallins (composed of two subunits, α A- and α B crystallins) are recognised as heat shock proteins (Hsps) and function as 'molecular chaperones' (Arrigo and Simon, 2010). Hsps are synthesised in response to diverse stressors such as elevated temperatures, hypoxia, inflammation and ischaemia (Lindquist and Craig, 1988). Their role is to prevent undesired protein aggregation by binding with mal-folded proteins that may arise in response to cellular stress protein translation (Andley et al., 1998; Voellmy and Boellmann, 2007). The α A- and α B-crystallins function as chaperones not only within the lens but also within other areas of the body. α A-crystallin is expressed mainly in the lens, whereas α B-crystallin exists in heart, skeletal muscle, skin, lung and the central nervous system (Graw, 2009). Indeed, the absence of crystallins normally found in the lens, brain, heart, skin, and skeletal muscle is associated with ageing phenotypes (Andley, 2008; Graw, 2009).

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Point mutations in the *Cryaa* gene, encoding α A-crystallin causes hereditary cataract (Graw, 2009). The functional consequence of the mutant protein may be reduced chaperone activity (Kumar et al., 1999) and a weaker anti-apoptotic effect (Mao et al., 2004). Mutations in the *Cryab* gene, encoding α B-crystallin, are associated with neurological, cardiac and muscular disorders (Graw, 2009). α B-crystallin has also been implicated in the pathogenesis of Alzheimer's disease (Mao et al., 2001), as α B-crystallin is present in the limbic and paralimbic regions, areas which are commonly affected in Alzheimer's disease. α B-crystallin may have a chaperone effect against aggregation of the disease-related amyloid fibrils (Ecroyd and Carver, 2008). Sarcopenia is the loss of skeletal muscle mass and strength, and a feature of senescent organisms. Studies of aged rat gastrocnemius muscle (an animal model of sarcopenia) were compared to young adult muscle from 3-month old rats. Ageing skeletal muscle showed a drastic increase in α B-crystallin and another Hsp, cvHsp/HspB7 (Doran et al., 2007). The upregulation of these Hsp may represent an auto-protective mechanism in degenerating muscle fibres. Crystallins therefore are not only involved in regulatory, cell survival roles within the eye but also play important roles in several other organs. It has therefore been suggested that cataract might be viewed as a 'bio-indicator' for less obvious, more severe age-related disorders (Graw, 2009). Lens transparency has also been described as a possible primary ageing phenotype, distinct from cataract, which may instead represent a disease phenotype (Sanders et al., 2011) (see section 8.5). The *Cryaa* and *Cryab* genes are just two examples of genes associated with hereditary cataract; other examples include the connexin (gap junction proteins) genes and those genes for syndromic cataract (Shiels et al., 2010). For a comprehensive overview, the reader is recommended to visit the *Cat-Map* database, an online chromosome map and reference database for cataract in humans and mice (<http://cat-map.wustl.edu/>). A few genes have been directly related to age-related cataracts. Genetic risk factors may account up to 75% of the heritability for age-related cataract (Hammond et al., 2001; Hammond et al., 2000a; Heiba et al., 1995). Variations in genes linked with inherited cataract have been associated with age-related cataract, including *EPHA2* (Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011). Additionally, there are variations in genes not directly associated with inherited cataract that may be associated in age-related cataract, such as the genes that function in antioxidant metabolism (*GSTM1*; *GSTT1*) (Siresha et al., 2012). The reader is referred to the *Cat-Map* database for contemporaneous information and data.

5. Glycation end products – their role in the eye and the ageing phenotype

Advanced glycation end products (AGEs) are a heterogenous group of macromolecules formed by the non-enzymatic glycation of proteins, lipids and nucleic acids (Semba et al., 2010). Reducing sugars such as glucose react with amino groups in proteins, lipids and nucleic acids. This occurs via the Maillard reaction with the formation of a Schiff base between glucose and ϵ -amino groups (e.g. lysine or arginine) that slowly rearrange to form relatively stable Amadori adducts (Glenn and Stitt, 2009). The

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most widely known Amadori product is the modification of haemoglobin (HbA1c), which is used clinically to monitor exposure to elevated blood glucose levels in the context of diabetes. Humans are exposed to two main sources of AGEs. Exogenous AGEs are ingested from foods, and formed when food is processed at elevated temperatures such as deep-frying, roasting, grilling and in the preparation of commercial foods (e.g. breakfast cereals, processed cheeses). Endogenous AGEs are formed in the body and production may be accelerated in diabetes and during normal physiological ageing.

AGE adducts are irreversible and form covalent cross-links with proteins. AGEs bind with the receptor for advanced glycation end products (RAGE); this evokes downstream inflammatory responses and oxidative stress that could play a role in age-related diseases (Schmidt et al., 2000; Yan et al., 2007). Ligand binding with RAGE leads to increased reactive oxygen species, upregulation of inflammatory cytokines, adhesion molecules and of its own receptors via NF- κ B (Bierhaus et al., 2001; Semba et al., 2010). AGEs have been implicated in accelerated ageing by instigating a strong inflammatory response. Activation of RAGE by various ligands is associated with several age-related conditions such as Alzheimer's disease (Takeuchi and Yamagishi, 2008), atherosclerosis (Basta et al., 2004), skin ageing (Lohwasser et al., 2006) and sarcopenia (Snow et al., 2007). Using a murine model of AMD, glycation altered proteolysis has been suggested as a pathobiologic mechanism that links ageing, dietary glycaemic index and age-related disease in non-diabetics (Uchiki et al., 2012).

Ageing of ocular tissues has been linked to the presence of AGEs in the cornea, the lamina cribrosa of the optic nerve head and the retina and lens. AGE adducts have been detected within the lamina cribrosa of the optic nerve, and it is postulated that AGE-mediated cross-linking at this location may reduce flexibility within the tissue matrix and induce age-related optic nerve damage, a characteristic feature of glaucomatous optic neuropathy (Albon et al., 1995; Glenn and Stitt, 2009). AGE accumulation has also been demonstrated immunohistochemically within human glaucomatous optic nerve and retina, and associated with ganglion cell degeneration, also characteristic of age-related glaucoma (Tezel et al., 2007). RAGE is also upregulated in the optic neuropathy associated with Alzheimer's disease (Wang et al., 2009). AGEs can accumulate in the retinal pigment epithelium (RPE), and immunoreactive AGEs have been demonstrated in drusen from ageing eyes (Schutt et al., 2003) and at elevated levels in patients with AMD (Howes et al., 2004). RAGE has also been observed in AMD (Hammes et al., 1999) and AGE accumulation on Bruch's membrane is associated with age-related dysfunction of the RPE (Glenn et al., 2008). A RAGE-mediated pro-inflammatory response within the outer retina is thought to be important in the context of retinal ageing and para-inflammation (Glenn and Stitt, 2009). In an *in vivo* model of retinal ageing, presence of RAGE ligands is associated with chronic inflammation within the outer retinal layers (Tian et al., 2005). Lens crystallins are subject to glycation and AGE cross-linking (Nagaraj et al., 2010). Glycation occurs at a higher rate during diabetes in both human (Garlick et al., 1984) and rat lenses (Yano et al., 1989). The accelerated cataract formation in

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diabetes is related to glycaemic control (Larsen et al., 1989), supporting this view. A stable AGE product, CML, is present in human lenses (Ahmed et al., 1986), and higher levels of CML are seen in cataractous lenses compared to age-matched controls (Franke et al., 2003). Accumulation of the AGE argpyrimidine (the end product of a protein modification at the arginine residue by methylglyoxal (MGO)) increases apoptosis of lens epithelial cells *in vitro* and *in vivo* (Kim et al., 2012). Trabecular meshwork cells play an important role in the regulation of aqueous outflow and open-angle glaucoma. AGEs have been found to not only decrease cell survival by inducing apoptosis, but also promotes the senescence of trabecular meshwork cells accompanied by increased oxidative stress (Park and Kim, 2012).

6. Mitochondrial dysfunction in the eye and age-related conditions

Mitochondrial damage can occur due to inherited mitochondrial mutations (e.g. Leber's hereditary optic neuropathy), but stochastic, cumulative oxidative damage is also likely to play a role in age-related disorders (Jarrett et al., 2010). Mitochondrial DNA (mtDNA) is maternally inherited. Due to the polyploid nature of the mitochondrial genome, wild-type and mutated mtDNA may coexist in individual organelles, or within a single cell or tissue; this condition is known as heteroplasmy. Most mtDNA mutations related to diseases are found in heteroplasmy, and the levels of normal and mutated mtDNA can vary considerably between cells of the same tissue (Jarrett et al., 2008). Specific mtDNA "polymorphisms" have been classified into a number of specific mitochondrial haplogroups (Torroni et al., 1996). Mitochondrial DNA haplogroups have recently been shown to be associated with a number of neurodegenerative conditions, such as Parkinson's disease (Autere et al., 2004; van der Walt et al., 2003).

The mitochondrial theory of ageing is related to the production of reactive oxygen species (ROS). Harman (2009) noted that oxygen free radicals are formed routinely during endogenous metabolism, and these may be involved in the ageing process. Other ROS can arise endogenously from the activity of cellular (particularly mitochondrial) enzymes such as peroxisomes, lipoxygenases, and cytochrome P450, as well as the respiratory activity of mitochondria themselves. Exogenous ROS can arise from UV light, visible light, ionising radiation and environmental toxins. Mitochondria play an important role in maintaining the redox balance that is crucial for cellular homeostasis. Production of ROS such as the hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($\text{OONO}\cdot$) is balanced with the production of antioxidants (such as superoxide dismutase, SOD), and other repair systems within the mitochondria (Brennan et al., 2012; McCord and Fridovich, 1969). Oxidative stress occurs when there is an imbalance between cellular ROS production and the ability of the cell to regulate ROS levels and repair damage caused by ROS (Brennan et al., 2012). Harman (1972) proposed that mitochondria play a key role in ageing. The theory proposes that ROS production leads to an accumulation of mitochondrial DNA (mtDNA) mutations, leading to mitochondrial dysfunction, and subsequent increased ROS production. ROS are thought to play an integral role in many age-related diseases, acting as the

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main drivers of ageing. The ROS-mediated damage is thought to have a causative role in the pathogenesis of several age-related conditions such as Alzheimer's disease, Parkinson's disease, hereditary spastic paraplegia and cerebellar degenerations (Beal, 2005).

mtDNA contains the genes encoding the proteins of the electron transport chain and those required for protein synthesis. Mitochondrial dysfunction may arise through changes in the mitochondrial genome or mutations in the nuclear genome affecting proteins needed by mitochondria. The replication and repair mechanisms of the mitochondrial genome are distinct from those of the nucleus, and continue in post mitotic cells such as the RPE and photoreceptors (Jarrett et al., 2010). mtDNA is thought to be more sensitive to ROS and other insults compared to nuclear DNA (Yakes and Van Houten, 1997). Mitochondria are less able to undergo DNA repair compared to nuclei; however, DNA repair mechanisms do exist including base excision repair (Jarrett et al., 2008). Other methods of mitochondrial protection include the presence of repair enzymes such as the glutathione system and thioltransferase system. These enzymes act as reducing agents to repair oxidative damage by maintaining the redox balance (Brennan et al., 2012). Antioxidants (e.g. methionine, cysteine, vitamins C and E) can directly scavenge ROS, and act as 'sinks' for ROS without permanent adverse consequences. The methionine sulfoxide reductase repair system is also believed to have a role in protection against oxidative stress; they are a family of enzymes that reduce methionine sulfoxide back to its reduced form (Brennan and Kantorow, 2009). The MsrA enzyme has been associated with the ageing process, with MsrA knockout mice showing a 40% reduction in lifespan compared to wildtype (Moskovitz et al., 2001). Loss of Msr activity has also been implicated in the pathogenesis of age-related cataract (see below).

6.1 Mitochondria and the eye

Tissues with high energy demands have higher numbers of mitochondria per cell (Brennan and Kantorow, 2009). The retina is the most oxygen consuming tissue in the body (Rattner and Nathans, 2006); mitochondria are found throughout the tissue, with the highest concentration within the retinal photoreceptors (Brennan and Kantorow, 2009). Mitochondria are crucial for ocular function, representing the major source of a cell's supply of energy (Jarrett et al., 2010). The ocular tissues are subject to highly oxidizing microenvironments with constant exposure to damaging agents such as visible light and/or UV wavelengths, environmental agents (e.g. tobacco smoke) and radiation. Oxidative stress and subsequent mitochondrial dysfunction may play an important role in cataract formation. Oxidative stress in the lens has been proposed as a model for understanding the role of oxidative stress systems in age-related human disease (Brennan et al., 2012). Lens crystallins must remain in a homogenous state to maintain lens clarity, and for this to occur, redox balance must be maintained. Mitochondrial dysfunction and imbalance of ROS have been implicated in cataract formation (Brennan et al., 2012), as has depletion of the reduced form of the GSH system, which helps

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to maintain the lens in a transparent state. (Calvin et al., 1991; Spector, 1995). Proteins in the cataractous lens are oxidised and form insoluble aggregates (Lou, 2003), for example an increase in oxidised methionines is seen with age (Truscott, 2005). The Msr enzymes may play an important role in age-related cataract formation, as oxidation of methionines and cysteines appears to play a significant role in the development of cataract (Brennan and Kantorow, 2009; Brennan et al., 2010). Decreased expression of base excision repair (BER) enzymes occurred with increasing age in the lenses of aged rats, suggesting that increased mtDNA damages causes a 'vicious cycle' of oxidative stress leading to accumulation of mtDNA mutations and cataract formation (Zhang et al., 2010). Genetic polymorphisms of BER enzymes have also been associated with age-related cataract in a Chinese population (Zhang et al., 2012). Clinically, antioxidants in the form of vitamins C and E have been investigated in epidemiological studies of cataract. Plasma measures of vitamins C and E taken at the eye examination were inversely associated with the prevalence of nuclear opacities in US and Indian populations (the latter an anti-oxidant depleted sample) (Dherani et al., 2008; Jacques et al., 2001).

Animal and clinical studies also support a role for mitochondrial genomic dysfunction and defects in ROS systems in AMD (Brennan and Kantorow, 2009; Jarrett et al., 2010). Histologically, there is a decrease in number and area of RPE mitochondria with increasing age, with significant increases in the eyes of AMD donors (Feher et al., 2006). There is also a significant association between mtDNA haplotypes and AMD prevalence (Canter et al., 2008; SanGiovanni et al., 2009). The variants associated with a higher risk for AMD occur within the mt coding region that encodes for proteins of the electron transport chain, suggesting that the resultant altered biogenetics may be expressed clinically in macular RPE as maculopathy. MtDNA is preferentially damaged with AMD progression in all regions of the mt genome (compared to just one region in 'physiological' ageing), beyond levels found in age-matched controls (Kaarniranta et al., 2011). Total mtDNA decreases with age, however there is no significant AMD-dependent change. Increased mtDNA deletions have also been noted in human and rodent retinas (Barreau et al., 1996; Wang et al., 2008). Mitochondrial dysfunction is also thought to contribute to the pathogenesis of primary open angle glaucoma (POAG), where a major risk factor is ageing (Leske et al., 2007). POAG has been associated with mtDNA mutations (Abu-Amero et al., 2006), and oxidative stress has been implicated in the pathogenesis of glaucoma (Tezel, 2006). The link between increasing age and prevalence of glaucoma suggests that ageing may increase the vulnerability of the optic nerve to stressors such as oxidative stress. Oxidative stress with increasing age may also be related to neurodegeneration in glaucoma (Lee et al., 2011).

It has been hypothesized that a vascular role is involved in glaucoma pathophysiology particularly as reduced ocular perfusion pressure is a risk factor for the disease (Leske, 2009). Cherecheanu et al., (2012) propose that the primary insult occurs at the optic nerve head. Increased intraocular pressure and ischemia at the post-laminar optic nerve head affects retinal ganglion cell axons. Following this primary insult retinal ganglion cells function at a reduced energy level and are sensitive to secondary

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insults. These secondary insults may happen if ocular perfusion pressure falls below the lower limit of auto-regulation. The mechanisms appear to involve vascular endothelial dysfunction and impaired astrocyte-vessel signalling.

Prohibitins are ubiquitous highly conserved membrane-associated proteins that can stabilize the mitochondrial genome. They are seen as 'mitochondrial chaperones', modulating the response to oxidative stress (Nijtmans et al., 2000). Proteomic *in vivo* mice models suggest that prohibitin is involved in oxidative stress signalling in the retina and RPE, and could act as a biomarker protein. Prohibitin levels were found to be perturbed in aged and diabetic rodent eyes (Lee et al., 2010), while down-regulation of prohibitin occurred in the RPE. Upregulation of prohibitin occurred in the ganglion cell and inner nuclear layers of diabetic eyes, suggesting links between prohibitin and diabetic pathways when accompanied by oxidative stress and inflammation. Prohibitin could be an oxidative stress biomarker in ageing and diabetes, and a possible therapeutic target (Schrier and Falk, 2011).

7. Epigenetic mechanisms and their role in age-related eye disease

Epigenetics is the study of phenotypic changes that may occur in a cell independent of changes in the underlying genetic code (Rando, 2010). Epigenetics originally related to the study of how genotypes give rise to phenotypes through programmed changes during embryonic development (Waddington, 1942). Epigenetics refers to both heritable changes and any mechanism that changes genes without changing the nucleotide sequence, and may be conceptualized as a developmental cascade where earlier or concurrent events act sequentially during cell proliferation and maturation to influence the developmental destiny of the cell (Kahn and Fraga, 2009). Genetic programmes for development, differentiation, and response to stress at a cellular or organ level can be altered by epigenetic modifications. The most well studied epigenetic regulatory mechanisms include covalent chemical modification of DNA (methylation), chromatin (covalent histone post-transcriptional modifications) and non-coding RNAs (miRNAs). These mechanisms are ultimately related to the regulation of gene expression and chromatin structure.

The vertebrate eye is thought to be an excellent model to study epigenetic mechanisms of gene regulation as regulatory genes and their hierarchies in the eye are among the best understood in mammalian systems (Cvekl and Mitton, 2010). The epigenome has also been described as a 'lens' through which genomic information is filtered, which deteriorates with age due to loss of integrity of genetic information and environmental stresses (Rando and Chang, 2012). The epigenetic 'lens' is thought to be essential for establishing the aged phenotype and may be the target for reprogramming the ageing process towards rejuvenation instead (Rando and Chang, 2012). The management of ocular disease in future may also employ manipulation of epigenetic pathways (Nickells and Merbs, 2012).

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7.1. DNA methylation

DNA methylation consists of the addition of a methyl group to the aromatic ring of a single DNA base (Calvanese et al., 2009) and is generally associated with silencing of gene expression (Kahn and Fraga, 2009). Genomic global DNA methylation decreases with age (DNA hypomethylation), first demonstrated by Berdshv et al in spawning humpbacked salmon (Berdyshev et al., 1967). However, hypermethylation of specific loci (CpG island promoters) have also been described during ageing (Fraga and Esteller, 2007; Oakes et al., 2003). Global hypomethylation and aberrant promotor hypermethylation have also been linked to cancer (Esteller, 2008). DNA methylation patterns can also change as a result of ageing or diet (Calvanese et al., 2009). DNA methylation is tissue-specific and may be cell type-specific, thus a certain DNA methylation pattern found in one tissue may not reflect patterns in other tissues (Michels, 2010). It is hypothesized that in age-related eye disease, environmental factors in combination with ageing can influence DNA methylation of critical genes required for normal function of the visual system (Cvekl and Mitton, 2010). The link between epigenetic mechanisms and the environment is highlighted in the external ocular tissues that are directly exposed to environmental toxins. Pterygia are abnormal proliferations of the conjunctiva adjacent to or encroaching onto the cornea, and aberrant DNA methylation has also been demonstrated in several key genes associated with wound healing processes in pterygia (Riau et al., 2011). Hypermethylation of the p16 gene promoter (which encodes for a negative cell cycle controller) and expression of a DNA methyltransferase is strongly correlated to reduced expression of the p16 protein (Chen et al., 2006). DNA methylation is also associated with altered gene expression in AMD - comparison of DNA methylation together with mRNA levels between AMD versus normal retinas showed significant differences in the *GSTM1* and *GSTM5* gene promoters, suggesting they undergo epigenetic repression in AMD RPE/choroid, which may increase susceptibility to oxidative stress (Hunter et al., 2012).

7.2 Micro-RNAs

Non-protein coding RNAs, microRNAs (miRNAs), are endogenous, small, non-coding, regulatory RNAs, approximately 22 nucleotides (nts) in size. miRNAs are regulatory molecules of chromatin structure and transcriptional elongation (Cvekl and Mitton, 2010; Xu, 2009). They can induce the breakdown of the targeted mRNAs and/or inhibition of translation from the mRNAs. Approximately 30% of protein-coding genes are regulated by miRNAs, and miRNAs play important roles in cellular proliferation, differentiation, and cell death. Mutations in miRNAs and/or the target sites in the transcripts of their downstream target genes, and dysregulation of miRNA biogenesis can result in a wide variety of diseases, including cancers.

The miRNA transcriptomes of the retina, lens, and cornea have been established. Many miRNAs show unique tissue-specific and developmental stage-specific expression patterns, suggesting potential

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unique functions in the retina and other ocular tissues (Xu, 2009). For example, several miRNAs are involved with retinal development (Huang et al., 2008). In addition, Dicer, a ribonuclease essential for miRNA processing, is necessary for the developmental changes of retinal progenitor cells (Georgi and Reh, 2010). Dicer is also highly expressed in developing mouse cornea and lens, where targeted deletion of Dicer disrupts lens morphogenesis, corneal epithelial stratification, and whole eye development (Li and Piatigorsky, 2009). The retina is amenable to experimental manipulation, and genetic manipulations that disrupt visual system development or physiology but which do not cause premature death, and thus permit detailed study of the phenotypic consequences (Sundermeier and Palczewski, 2012). For example, miRNAs are involved in the pathogenesis of retinopathy in diabetic rats by modulating multiple pathogenetic pathways (Kovacs et al., 2011) and miRNA profiling of ischemic retinas reveals that some may be upregulated whereas others are down-regulated which suggest a role in the pathogenesis of ocular neovascularisation as seen in AMD and diabetic retinopathy (Shen et al., 2008). During ageing, dysregulated expression of miRNAs can occur which may contribute to the pathogenesis of several age-dependent disease states (Lanceta et al., 2010). For example, distinct expression profiles of miRNAs in the central epithelium of transparent and age-related cataractous human lenses have been detected suggesting differential expression of miRNAs has a potential role in lens development and/or cataract formation (Wu et al., 2012). A local increase of the let-7b miRNA may represent a risk factor in the formation of age-related cataracts (Peng et al., 2012). The miRNA *MIR204* is also thought to play an important role in the regulation of multiple functions in human trabecular cells including apoptosis, accumulation of damaged proteins, and expression of inflammatory mediators (Li et al., 2011)

7.3 Histone modifications and the role of sirtuins in the retina

Histone modifications occur at the level of the nucleosome. The nucleosome is the fundamental unit of chromatin, which contains an octamer of histones. DNA makes approximately 1.67 turns around this octamer unit. Some histone tails are not embedded fully in the nucleosome structure, which permits access to histone modifying enzymes, and subsequent post-translational modifications (PTMs). Histone PTMs work in concert to form a 'histone code' that is read by other proteins to regulate gene expression (Willis-Martinez et al., 2010). Histone modifying enzymes are thought to have an important role in ageing, particularly histone deacetylases (HDACs), which are important for signal regulation. The class III HDACs are NAD⁺ dependent proteins called sirtuins. They are orthologues of the yeast transcriptional regulator SIR2. In humans, seven Sirtuins have been identified (Sirt1–7) (Frye, 2000; Michishita et al., 2005), all with unique characteristics, functions, and localisations allowing increased functional complexity than in yeast through these distinct orthologous forms. Sirtuins are involved in chromatin remodelling, transcriptional silencing, mitosis and control of lifespan (Calvanese et al., 2009). SIRT1 expressed in mammals is the orthologue displaying the strongest homology to Sir2. In yeast Sir2

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deletion shortens replicative lifespan, and an extra copy increases it, suggesting the Sir2 family plays a role in ageing (Kaeberlein et al., 1999). SIRT1 is expressed in most tissues and down-regulated in senescent cells and ageing (Sasaki et al., 2006; Sommer et al., 2006). SIRT1 may have a role in regulating DNA stability, and promotion of cell survival via anti-apoptotic processes (Ozawa et al., 2010). SIRT1 may confer protection against age-related metabolic and inflammatory processes (Donmez and Guarente, 2010). Sirtuins have a pivotal role in the expansion of lifespan in lower organisms via caloric restriction, better fuel utilisation and ultimately reduced production of ROS (Kaeberlein et al., 1999; Muller et al., 1980; Rogina and Helfand, 2004). Sirt1 also acts as a 'tumour keystone' (McGuinness et al., 2011), where its level and action maintain a fine and delicate balance between suppression and promotion of oncogenesis, by providing both tumour suppressor and tumour promoter functionality (Deng, 2009). Sirtuins have not yet been extensively investigated in the eye, but SIRT1 is expressed throughout the retina (Greiss et al., 2008; Jaliffa et al., 2009). Reduced expression of Sirt1 has been found in a mouse model of retinal degeneration, possibly reducing the functions of Sirt1 in DNA repair mechanisms which may be important in the pathogenesis of age-related conditions (Jaliffa et al., 2009).

7. 5 Epigenetic epidemiology and age-related eye disease

Epigenetic epidemiology is the study of the association between epigenetic variation and the risk of disease in humans (Michels, 2010). Epigenetic modifications are stable and heritable but can be modulated by many factors, including physiological and pathological conditions and the environment (Calvanese et al., 2009). In this way environmental factors could influence gene expression and phenotype and provide a molecular link between ageing and the environment (Feil and Fraga, 2011). Epigenetic traits such as CpG methylation can be used as markers in epidemiologic studies and comparisons can be made of the degree of global methylation, DNA methylation patterns, or gene-specific methylation between population groups defined by the presence or absence of a specific exposure or disease. For example, global DNA hypomethylation has been observed in the most socio-economically deprived individuals in a Scottish population cohort (McGuinness et al., 2012).

Epigenetic changes accumulate over time, and can account for observed inter-individual differences in expression and phenotype (Thompson et al., 2010). Twin studies of monozygotic twins have provided useful insights, demonstrating the influence of non-genetic factors on the generation of epigenetic changes over time (Fraga et al., 2005), which may explain phenotypic discordance between MZ twins (Calvanese et al., 2009). In another study of elderly MZ twin pairs who lived apart, greater phenotypic discordance was associated with greater epigenetic differences than young and phenotypically similar MZ twins reared together (Fraga et al., 2005). A genome-wide analysis of DNA-methylation patterns in MZ and dizygotic (DZ) twins demonstrated significant epigenetic differences in both groups (Kaminsky et al., 2009). A heritable component to AMD has previously been demonstrated (Hammond et al.,

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2002), with genetic factors explaining 31-71% of disease severity. Further evidence of the epigenetic differences between twins has been reported in AMD: in another study of MZ twins with different AMD phenotypes, heavier smoking was significantly associated with advanced AMD, whereas dietary intakes of vitamin D, methionine and betaine were associated with smaller lesions (Seddon et al., 2011). These findings suggest that smoking and some nutritional factors may induce epigenetic changes that modify gene expression and hence phenotype (Seddon et al., 2011).

8. Ophthalmology and translational research

Ophthalmology lends itself to translational research. Examples of basic age-related research translated clinically include the anti-VEGF (vascular endothelial growth factor) drug ranibizumab used as a therapy for 'wet' age-related macular degeneration (AMD) (Adamis et al., 1996; Aiello et al., 1995), a leading cause of irreversible blindness in elderly persons in developed countries which accounts for 9% of visual impairment worldwide (Klein et al., 2011; Ngai et al., 2011; Pascolini and Mariotti, 2012). Clinical trials showed that intraocular injection of ranibizumab improved vision in one-third of patients with 'wet' AMD (typically the AMD form least amenable to treatment), while stabilizing the condition in 90% of cases, in contrast to previous therapies that did not lead to clinically significant improvements in visual acuity (Brown et al., 2006; Rosenfeld et al., 2006). Another example of translational research is gene therapy, an example being transfer of the *RPE65* gene into the RPE of individuals with Leber's congenital amaurosis (LCA). This intervention can partially restore visual function (Bainbridge et al., 2008; Maguire et al., 2008). In addition, about a third of entries in the Online Mendelian Inheritance in Man database for which a clinical synopsis is provided, include a term referring to the structure or function of the eye (Sheffield and Stone, 2011). Ocular stem cell therapy in animal eye models also has translational applications: endothelial cell precursor cells (EPCs) have been successfully used in retinal degeneration models to promote photoreceptor survival as well as promoting vascular repair in models of ischaemic retinopathy (Otani et al., 2004; Ritter et al., 2006; Sasahara et al., 2008). In addition, some of the first applications of nanotechnology and nanomedicine have occurred in this field (Zarbin et al., 2010a, b). There is also growing interest in exploring the suitability of gene delivery strategies in ocular therapy as they may represent drug delivery mechanisms that occur within the CNS (Diebold and Calonge, 2010).

Ophthalmology and clinical research findings

Global estimates of blindness show that age-related cataract, AMD and glaucoma account for a large proportion of low vision and blindness (Pascolini and Mariotti, 2012; Resnikoff and Keys, 2012); these conditions also most prevalent in the elderly and contribute to overall disability in elderly people (Sousa et al., 2009). Several large-scale epidemiological studies have been performed globally, providing strong evidence for genetic susceptibilities to age-related eye disease. One such example is the *Y402H* complement H variant conferring a 2-fold higher risk of late-AMD per copy in individuals of European

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descent (Sofat et al., 2012); the Blue Mountains Eye Study conducted in Sydney additionally found that individuals with the Y402H variant had an increased likelihood of bilateral compared with unilateral involvement of AMD lesions (Pai et al., 2009). The association of *Y402H* with AMD was also confirmed in the Rotterdam Study, accounting for a substantial proportion of AMD particularly in the presence of environmental and genetic stimulators of the complement cascade (Boekhoorn et al., 2007; Despriet et al., 2006; Hofman et al., 2009). Genetic variants also contribute to AMD in the Chinese population (Tian et al., 2012). A global genome-wide association study for intra-ocular pressure (IOP), a risk factor for glaucoma, has identified two clinically relevant genes, *GAS7* and *TMC01*, involved in IOP regulation (van Koolwijk et al., 2012). A heritable component to age-related cataract has been established in twin studies (Hammond et al., 2000b), and single nucleotide polymorphisms (SNPs) associated with age-related cataract have been identified within the *GJA8* and *CRYBB3* genes in a German population-based study (Graw et al., 2011). Age-related eye disease is also associated with systemic comorbidities and lifestyle effects. For example, age-related cataract is associated with smoking, alcohol consumption and blood glucose control (Kanthan et al., 2010, 2011; Tan et al., 2008a; Tan et al., 2008b). Smoking and cardiovascular risk factors are also associated with AMD (Chakravarthy et al., 2010). Cataract prevalence is also reported as being higher in women (Graw et al., 2011), but may also reflect differing access to cataract surgery services in resource-constrained settings (Lindfield et al., 2012).

8.1. Ocular parameters that could be used in ageing research:

Clinically, the eye is relatively easy to examine compared to other organ systems and most ocular structures can be examined at a magnified view with minimal discomfort to the patient. There are few other locations in the body where cells, vasculature and neurological tissues are directly and readily visible. Several instruments not requiring ophthalmological expertise can provide objective measurement e.g. endothelial cell counts of the cornea, intra-ocular pressure, thickness of the retinal layers, the diameter of retinal vessels. Furthermore, visual function can be assessed using a variety of tests to measure visual acuity, contrast sensitivity, colour vision etc. Many of these parameters change with chronological age. Validated methods are listed in *Table 2*; a detailed description of parameters and their measurement follows and is represented in *Figure 1*.

8.1.1 Visual acuity

Large-scale epidemiological studies have shown that visual acuity decreases with increasing chronological age (Foran et al., 2003; Klein et al., 2006). It is probably one of the easiest parameters to measure and requires minimal training. Visual acuity can be measured using logarithmic visual acuity charts as per the protocol developed for the Early Treatment Diabetic Retinopathy Study (ETDRS) (Ferris and Bailey, 1996) where acuity is expressed as log MAR (the logarithmic value of the minimum angle of resolution). A grading system assigns a visual acuity score according to the number of letters

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read correctly. On this scale, a Snellen visual acuity of 20/20 corresponds to logMAR = 0.00; 20/40 corresponds to log MAR = 0.30, and 20/200 corresponds to log MAR = 1.00 (Bailey et al., 1991). The letter sizes on the chart progress in 0.10-log unit steps and there are five letters on each row and each letter is assigned a value of 0.02 log units. Results can either be given as number of letters read, or the Snellen equivalent. Near visual acuity can be measured in a similar manner.

8.1.2 Colour vision

Colour vision, which is a function of cone photoreceptors, is the ability to discriminate objects based on the different wavelengths (or frequencies) of light they reflect, emit, or transmit. Colour vision can result from retinal or optic nerve pathology, and decreases with age (Roy et al., 1991). The latter may reflect changes in the crystalline lens, the retina, or the pupil size, either separately or in combination (Nguyen-Tri et al., 2003; Roy et al., 1991). Colour vision can be measured in several ways: the Farnsworth-Munsell 100 hue test (FM100) consists of 85 coloured caps which represent the entire colour circle. Colour discrimination is assessed by asking the person being tested to place the caps in the appropriate order starting at a fixed colour, under standard illumination conditions (Mäntyjärvi, 2001). Total error score can be calculated and the square root of the total error score used in analyses. Simpler tests exist for example in the form of Ishihara colour plates, typically used to evaluate red-green colour blindness.

8.3 Contrast sensitivity

Contrast sensitivity is the visual ability to see objects that may not be outlined clearly or that do not stand out from their background. Contrast sensitivity decreases with increasing chronological age, even after adjusting for visual acuity (Hashemi et al., 2012; Nomura et al., 2003; Shandiz et al., 2011; Sia et al., 2012). Low contrast sensitivity can be more disabling than visual acuity loss (Leat et al., 1999), and is a better predictor of mobility performance than visual acuity (Marron and Bailey, 1982). Decreased contrast sensitivity is associated with ischaemic retinal disease and cataract (Shandiz et al., 2011; Shoshani et al., 2011) and may be a sensitive indicator of generalized ageing in the most elderly (Lott et al., 2010). Contrast sensitivity loss can be present even when visual acuity and fields are relatively intact (Elliott and Whitaker, 1992). The ability to detect objects of different sizes at lower contrasts is expressed as a contrast sensitivity function (CSF) and determines the person's contrast detection threshold, the lowest contrast at which a pattern can be seen. Contrast sensitivity scores, which are linear on a log scale, can be determined using a Pelli-Robson contrast sensitivity chart; lower logCS values reflect worse contrast sensitivity.

8.4 Corneal endothelium

The corneal endothelium consists of a monolayer of non-replicating, mosaic-like cells, which vary from 4 to 6 μm in thickness and span 20 μm in width, and have a stable and metabolically efficient hexagonal shape (Bourne and McLaren, 2004). Morphological characteristics of the endothelium evolve with

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ageing: some cells enlarge as others disappear. The monolayer can be viewed via specular microscopy using the technique of specular reflection (Figure 2) (Amann et al., 2003). Polymegethism (cell size) and polymorphism (geometric cell parameters) of the cells can be ascertained by this technique as well as the number of cells within a defined area, to give a measure of endothelial cell density (ECD) (Wörner et al., 2011). Endothelial cell density is negatively correlated with age (Laing et al., 1976; Sanchis-Gimeno et al., 2005; Wilson and Roper-Hall, 1982) and decreases throughout life at an average rate of about 0.6%/year (Bourne et al., 1997). Mean cell density is approximately 3400 cells/mm² at age 15 years declining to approximately 2300 cells/mm² by 85 years (Yee et al., 1985). However racial or geographic differences appear to exist (Hashemian et al., 2006; Rao et al., 2000; Snellingen et al., 2001; Yunliang et al., 2007). Endothelial cells from human corneas and mouse senescence models also show increased expression of senescence-related genes with increasing age (Song et al., 2008; Xiao et al., 2009). The gene encoding CDKN2A (p16^{ink4a}) (a negative cell cycle regulator) is thought to play a key role in the process of senescence of corneal endothelial cells (Song et al., 2008). Human corneal endothelial cells (HCECs) also exhibit signs of oxidative DNA damage: 8-hydroxy-2'-deoxyguanosine levels (8-OHdG; a marker of oxidative DNA damage) were significantly higher in the central endothelium of older donors than of young donors (Joyce et al., 2009). Human corneal endothelial cells have been demonstrated to have long telomeres throughout life (Egan et al., 1998), and it is suggested that their limited replicative ability does not result from critically short telomere lengths. The limited replicative potential of the endothelium has functional consequences as there is a minimal cell density that is compatible with a clear cornea (and therefore good visual acuity). More recent work suggests 'stress-induced premature senescence' (caused by exposure of cells to environmental stressors) leads to age-related and topographical reduction in replicative capacity and senescence characteristics (Konomi and Joyce, 2007), and that this mechanism (rather than critical telomere shortening) leads to lack of proliferation.

8.5 Lens opacities

The prevalence of lens opacities increases exponentially with age (Hodge et al., 1995), and the human lens is considered an ideal tissue for studying macromolecular ageing in man (Truscott and Zhu, 2010). Affected individuals have a higher mortality risk than those without lens opacities (Wang et al., 2001). Assessment of lens opacity for research purposes have used clinical grading systems such as the Lens Opacities Classification System (LOCS) III (Chylack et al., 1993) and the Age-Related Eye Disease Study (2001). However, these systems are subjective, requiring slit-lamp evaluation with comparison against standard photographs and are, therefore subject to observer bias with low interobserver and intraobserver reliability (Pei et al., 2008). Objective quantification of lens opacity would be ideal. The Pentacam (Oculus, Germany) is a recently developed 360-degree rotating non-contact camera Scheimpflug imaging system with a lens densitometry function which provides an objective, quantitative assessment of lens opacity on a continuous scale from 0 to 100%. The Pentacam measures the light

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scatter of the lens that becomes visible by illumination with blue light (wavelength 475nm) (Figure 3) (Kirkwood et al., 2009). The images of lens density acquired from the Pentacam have been validated against the LOCSIII system and repeatability of measurements and correlation with LOCSIII grading has been demonstrated (Grewal et al., 2009; Kirkwood et al., 2009; Pei et al., 2008); To date, the Pentacam has not been used in studies of biological ageing or to assess lens opacity as a putative biomarker of ageing, but has been used clinically. Recently, the phenotype of 'lens transparency' has been described as a possible biomarker of ageing, and it has been suggested that cataract (which refers to lens density or opacification on clinical assessment) may be viewed as a "clinical disease phenotype" (Sanders et al., 2011)).

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8.6 Amplitude of accommodation

Accommodation is the ability of the eye to change the refractive power of the lens to focus on objects at a range of distances. The point at which accommodation is maximally exerted is called the near point. The reduction of this ability in which the near point recedes further away from a comfortable distance for the task being undertaken (e.g. reading, close work) is called presbyopia (Abraham et al., 2010). As the lens protein α -crystallin decreases with age in the human lens, there is a steady increase in the stiffness of the lens centre (nucleus), and this loss of elasticity may account for the loss of accommodation and hence presbyopia (Glasser and Campbell, 1999; Truscott, 2009). The age at which an individual becomes symptomatic varies, and is related to the refractive state of the eye (Abraham et al., 2005). An ecological study suggested high average temperature can accelerate the onset of presbyopia (Miranda, 1979). The amplitude of accommodation (AA) is the amount of accommodation exerted to move the focus from the far point to the near point. It decreases from childhood from about 15 diopters (D) in early childhood to 1 D before the age of 60 years (Abraham et al., 2005). The near point of accommodation can be measured clinically using non-invasive methods ('RAF rule'), and from this AA can be calculated.

8.7 Retinal nerve fibre layer (RNFL) and retinal cells:

The RNFL is formed by expansion of the fibres of the optic nerve and is affected in conditions such as glaucoma, AMD and choroidal pathologies. Thinning of the RNFL is associated with older age (Chi et al., 1995; Kanamori et al., 2003), and has also been detected in HIV-infected individuals, compared to age- and gender-matched controls (Faria et al., 2010; Kozak et al., 2005). Thinning of the RNFL may be manifest functionally as deficits in visual field testing, colour vision and contrast sensitivity. RNFL can be assessed using optical coherence tomography (OCT), a non-invasive, non-contact method which gives a cross sectional image of the retina and its substructures in a real time mode and *in vivo* (Hee et al., 1995). RNFL thickness is calculated from the reflectivity distribution within the retina, using a special algorithm. OCT is used extensively in clinical practice to aid management of posterior segment conditions (Sakata et al., 2009) (Figure 1).

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Thinning of the RNFL in Alzheimer's disease has been observed particularly within the superior quadrants of the peripapillary area (surrounding the optic nerve head) (Berisha et al., 2007; Kesler et al., 2011; Lu et al., 2010; Paquet et al., 2007). Similar findings have also been noted in Parkinson's disease and spinocerebellar ataxias (Hajee et al., 2009; Pula et al., 2011). These parameters may also be of practical importance as OCT is a quick non-invasive procedure and faster acquisition is important for patients with cognitive impairment such as AD. The retrograde loss of nerve fiber layer tissue in the retina and optic nerve may be an early biomarker of Alzheimer's disease, and deficits in the RNFL and optic nerve may be the earliest sign of disease, even prior to damage to the hippocampal region that impacts memory (Valenti, 2011). OCT may be of particular value in research of conditions such as Alzheimer's disease as assessment is quick and does not require high levels of cognitive function.

Fourier domain OCT has been used to identify subclinical RNFL thinning in patients with Parkinson's disease (Garcia-Martin et al., 2012) and to quantify retinal neural loss in multiple sclerosis (Monteiro et al., 2012). The technique of autofluorescence can also be used to assess the health and integrity of the RPE which is altered in AMD (Caramoy et al., 2012). The current gold standard for retinal vessel visualization is fluorescein angiography, which is used widely in clinical practice (Bernardes et al., 2011; Lim et al., 2012). A non-invasive alternative to image blood vessels is based on functional Fourier domain OCT, namely non-invasive wide-field angiography (Blatter et al., 2012; Campbell et al., 2012). Using the technique of 'adaptive optics', which overcomes wavefront distortions produced as light passes through the pupil of the eye, it is now possible to image single cells such as cone photoreceptors, within the living retina (Kocaoglu et al., 2011; Williams, 2011). Adaptive optics scanning laser ophthalmoscopy has demonstrated that the density of cone photoreceptors decreases with increasing age in healthy human subjects (Song et al., 2011).

8.8 Retinal vessel calibre

Retinal vessel diameters decrease with ageing. After adjustments for gender, hypertension and body mass index, retinal arteriolar diameter decreases between 2.1- 4.8 μ m per decade increase in age, and venular diameter 2.0-4.1 μ m per decade increase in age in Australian (non-indigenous) populations (Leung, 2003; Wong, 2003). Retinal vascular calibre is a structural marker of microvascular changes in the fundus and is strongly associated with hypertension, diabetes, and other systemic vascular diseases (Wong et al., 2002a; Wong et al., 2002b). It has been proposed that advancing age promotes oxidative stress and endothelial dysfunction in the vessel walls (Ehrlich et al., 2009). Structural changes in blood vessels (affecting retinal diameter) and impaired autoregulation may promote ischaemic changes. Noxious stressors can further disrupt retinal blood flow and may initiate a para-inflammatory process (see section 3.1). Validated quantitative measurement of retinal vessel diameters is possible using semi-automated software applied to digitized fundus photographs (Wong et al., 2004). The

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caliber and pathology of cerebral small vessels is difficult to assess *in vivo*. Most non-invasive markers of vascular pathology are related to major blood vessels outside the brain and may not represent local cerebral abnormalities. The retinal vessels provide unique opportunities to study cerebral small vessel disease, because they share similar anatomy, physiology and embryology (Wong et al., 2001). Retinal venular dilatation is related to progression of cerebral small vessel disease (Ikram et al., 2006). Larger venular calibres are associated with an increased risk of dementia, in particular vascular dementia but not Alzheimer's disease (de Jong et al., 2011). The association between larger retinal venular calibres and dementia may reflect cerebral hypoperfusion and subsequent ischemia. A meta-analysis of over 20,000 middle- to older-aged individuals without diabetes confirmed that wider retinal venular caliber is independently associated with an increased risk of stroke events. No association with narrower retinal arteriolar caliber was observed (McGeechan et al., 2009). The microvascular changes occurring in the retinal vasculature may correlate with macrovascular systemic changes that are often not as easy to image or require interventional techniques. In relation to cardiovascular disease, larger retinal venular diameters are related to atherosclerosis, higher levels of total cholesterol, lower levels of HDL, higher levels of inflammatory markers (such as erythrocyte sedimentation rate and leucocyte count) and smoking (Ikram et al., 2004). A decreased arteriolar diameter is a risk factor, after adjusting for confounders, for developing hypertension within three to 10 years in normotensive individuals (Mimoun et al., 2009).

9. Conclusions

Several mechanisms have been postulated to explain the ageing process; none are comprehensive and indeed this reflects the hypothesis that ageing occurs at cellular, organ and physiological levels (Fulop et al., 2010). The eye provides an excellent model in which to further study mechanisms such as genetic control of lifespan and healthspan, oxidative stress, macromolecular ageing and glycation, as these processes are all well represented in the eye. The eye also provides an interface between molecular, epigenetic and cellular mechanisms and how they are manifest clinically. The eye is likely to continue to play an important role in translational research, as well as functioning as an excellent system for testing the Baker and Sprott criterion for validating biomarkers of ageing.

At present data are limited regarding the role of the eye as a model of ageing. Some parameters have been more extensively researched than others and are likely to fulfil Baker and Sprott's criteria better than others. For example, retinal vessel calibre correlates with age and would be an ideal candidate for further research to investigate if it does fulfil the criteria as an ageing biomarker. Lens transparency can now be measured objectively and as cataract has already been established as a biomarker of morbidity and mortality, it may be that lens density can serve as a marker of healthy ageing. In comparison, parameters such as endothelial cell count and the retinal nerve fibre layer probably require more detailed study in larger population cohorts to establish their relationship with ageing in health and

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disease before warranting investigation as possible biomarkers of ageing. Many previously suggested ageing biomarkers do not fulfil the criteria as most of the markers under discussion are related not only to age, but also to disease, and thus none of these markers discussed is a 'pure' biomarker of ageing (Simm et al., 2008). It is not clear yet if this may affect some of the proposed ocular parameters. The authors therefore propose a research agenda to establish whether the eye may be a suitable research model (*Box 1*):

It is likely that biomarkers of ageing will act in synchrony, so that for example, biomarkers in peripheral blood lymphocytes (such as telomere length) reflect ageing in terms of a general measure of 'miles on the clock', and other biomarkers may provide more organ-specific information. Ultimately the purpose of developing an optimal model of ageing is to understand the mechanisms that permit healthy ageing in the face of age-related stressors, and to subsequently develop and assess potential interventions to increase or maintain healthspan, to enhance function and well-being, and to delay frailty and age-related disability. This should go some way in understanding the complex differences in the health and functional status of older populations that are now occurring at a global level, and assist with planning of public health interventions and resource delivery, as well as predicting clinical outcomes at the individual level.

Box 1:

Research agenda to assess the eye as a model of ageing and validation of BoA

1. Cross-sectional studies of the ocular parameters that have not been fully investigated as biomarkers of ageing e.g. endothelial cell count, amplitude of accommodation, contrast sensitivity. It is known that these parameters change with increasing chronological age in the eye, but associations with other putative biomarkers of ageing or systemic age-related parameters have not been assessed. Studies will need to be performed in cohorts of varying ages (not just elderly populations) to fully understand the influence of stressors and disease, as well as the pattern of aging trajectories for the different ocular parameters.
2. Longitudinal studies of well-defined ocular parameters in parallel with other biomarkers of ageing to verify and validate their use (e.g. Pentacam lens measurement with telomere length)
3. Elucidate a panel of biomarkers that can be further validated in longitudinal studies. In a cohort of 85-year olds in northeast England, 74 candidate biomarkers were evaluated, and 10 were identified as possible biomarkers of ageing (including vitamin D status, systolic blood pressure and hand grip strength), however no ocular parameters were assessed (Martin-Ruiz et al., 2011).
4. In terms of a 'healthy ageing phenotype', longitudinal measurements of e.g. lens density, retinal nerve fibre layer, retinal vessel calibre should be taken from early adult life or even younger in order to

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characterise the development of biological capital as well as ageing trajectories in terms of a 'life course' approach (Kuh, 2007).

5. Combine validated ocular parameters with other simple assessments of physical capability (e.g. grip strength, walking speed) to form a 'healthy ageing index' that could be used to predict risk of morbidity or mortality.

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Figure legends

Figure 1: Anatomy of the eye and examples of measurement of age-related parameters that may have translational research applications

Figure 2: Endothelial cells viewed by specular microscopy – A; endothelial cell density (ECD) of 1745 cells/mm² in a 65-year old female. B; ECD of 3014 cells/mm² in a 30-year old female. Endothelial cells typically have a hexagonal shape; this changes with ageing due to the non-replicative nature of the cells.

Figure 3: Imaging of lens density using the Pentacam – A; lens appearance in a 35-year old female with a near-transparent lens. B; lens in a 65-year old female with a dense lens. Clinically this would be visible as a cataract.

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Ms. Ref. No.: ARR-D-12-00040

Table 1: How ocular ageing correlates with systemic ageing

Ocular Parameter	Correlation with ageing systemically	Example	Reference
Vessel calibre	Neurological	Retinal venular dilatation associated with dementia and CVA	de Jong et al., 2011; McGeechan et al., 2009
	Cardiovascular	Retinal venular dilatation associated with cardiovascular ischaemic changes Retinal arteriolar narrowing associated with hypertension	Ikram et al., 2004; Mimoun et al., 2009
Lens opacity	Mortality	Mixed lens opacities predictors of mortality after adjusting for confounders	West et al., 2000; Hennis et al., 2001
Lens transparency	Leukocyte telomere length (LTL)	Lens transparency associated with longer telomere length	Sanders et al., 2011
Retinal nerve fibre layer	Neurological	Thinning in Alzheimer's disease, spinocerebellar ataxias, Parkinson's disease	Lu et al., 2010; Hajee et al., 2009; Pula et al., 2011; Kesler et al., 2011
Amplitude of accommodation	Neurological	Impaired convergence – leading to difficulties reading and viewing close objects in Parkinson's disease	Almer et al., 2012
Visual function (including Visual acuity, contrast sensitivity, colour vision)	Mortality	Visual acuity and other 'non-standard' vision measures are predictors of mortality in community-dwelling elderly population	Lott et al., 2010
	Frailty	Poorer visual function associated with greater frailty Visual function biomarkers of ageing associated with increased risk of falls	Klein et al., 2003; Knudtson et al., 2009
	Neurological	Decreased contrast sensitivity and difficulties in visual perception in Parkinson's disease	Armstrong, 2011

Ms. Ref. No.: ARR-D-12-00040

Table 2: Ocular parameters, methods of measurement and the impact of ageing

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Anatomical site	Parameter	Method of measurement	Age-related changes
Cornea	Corneal endothelium	Specular microscopy	Reduced cell count
Lens	Lens opacity	Subjective grading systems Pentacam – lens densitometry	Opacification at different sites in the lens (cataract)
	Amplitude of accommodation	RAF rule to measure near point of accommodation	Reduced amplitude
Retina	Retinal nerve fibre layer (RNFL)	Optical coherence tomography	Thinner RNFL
	Retinal vessel caliber	Semi-automated retinal analysis software	Reduced diameter of arterioles and venules
	Retinal ganglion cells	Confocal scanning ophthalmoscopy	Apoptosis
	Retinal photoreceptors	Confocal scanning ophthalmoscopy	Reduction in cone photoreceptor density
Visual function	Visual acuity	Visual acuity chart (ETDRS)	All reduced
	Contrast sensitivity	Pelli-Robson chart	
	Colour vision	Farnsworth-Munsell 100 hue test	

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Highlights

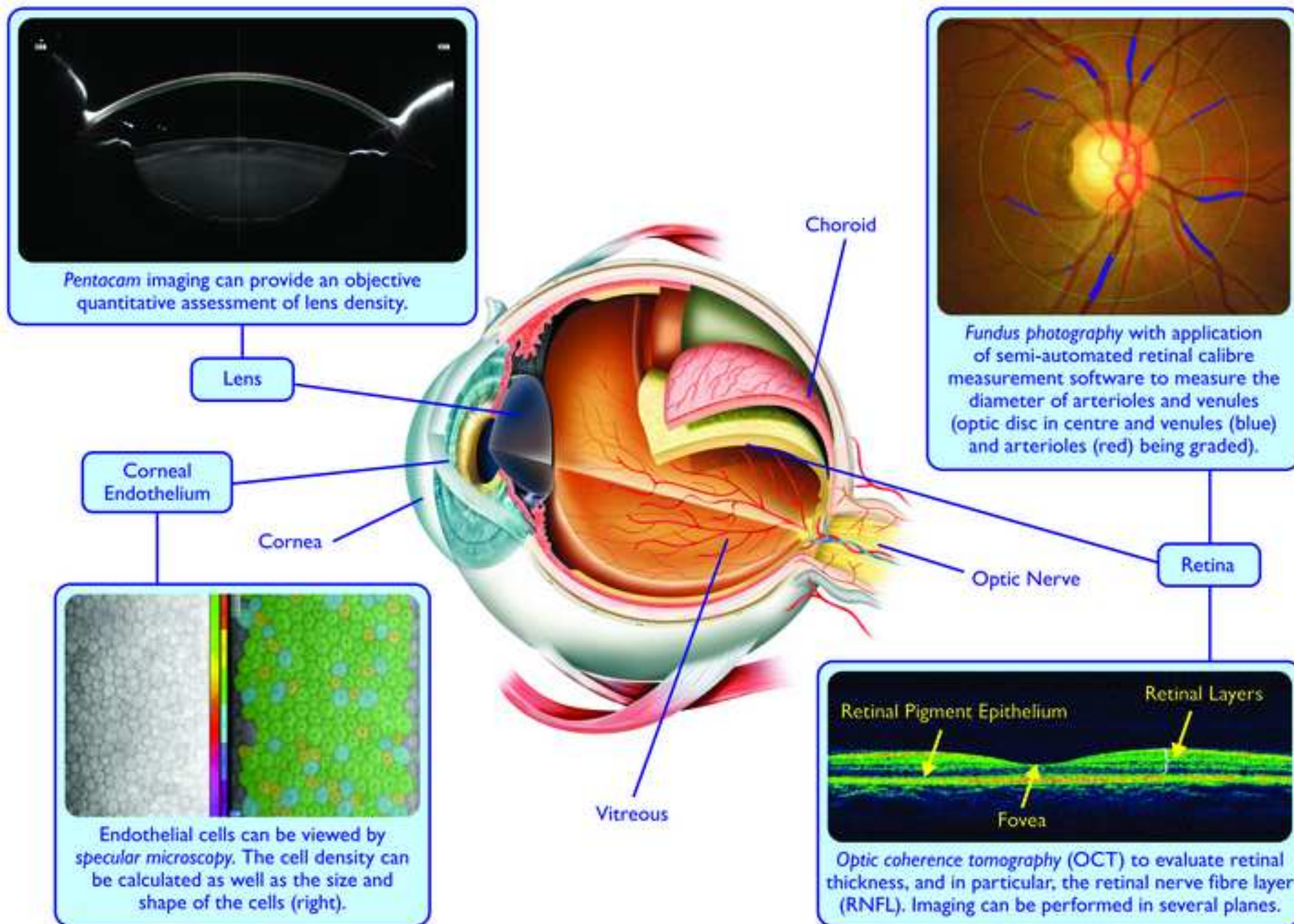
- The eye and visual system may be a uniquely useful site as a model of ageing
- Oxidative stress, macromolecular ageing and glycation are well represented in the eye
- Retinal vessel calibre and lens transparency may be novel biomarkers of ageing

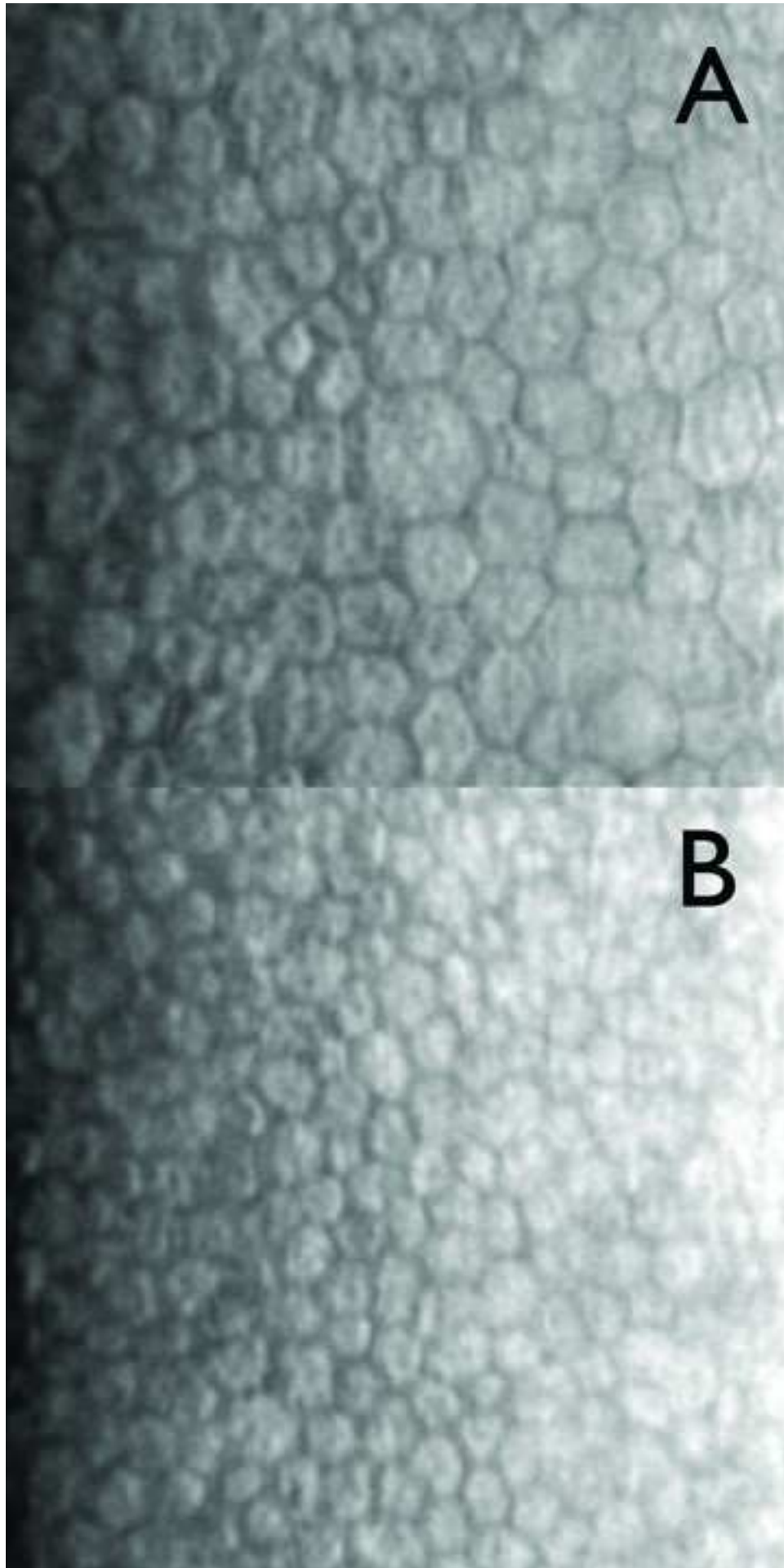
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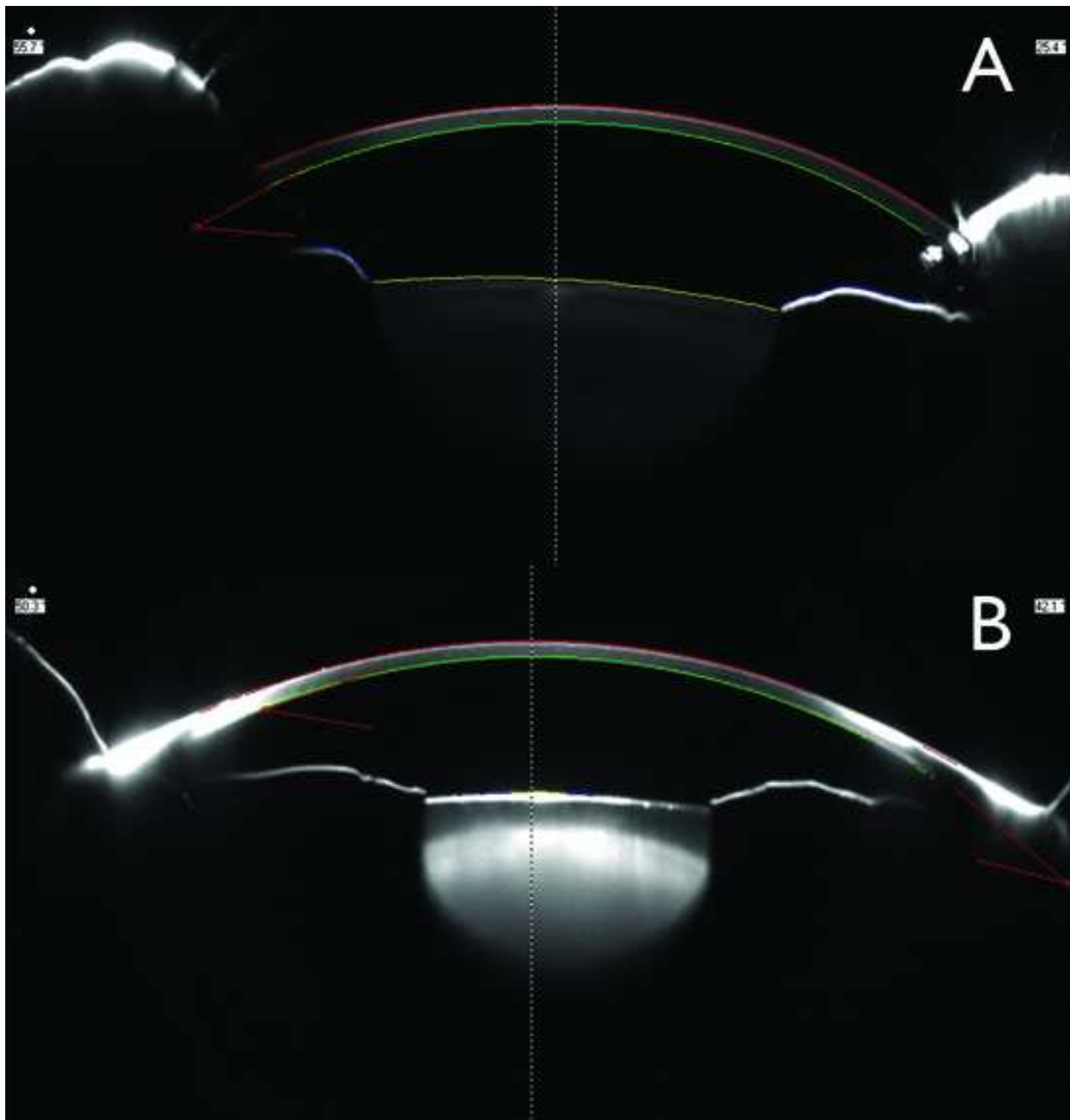
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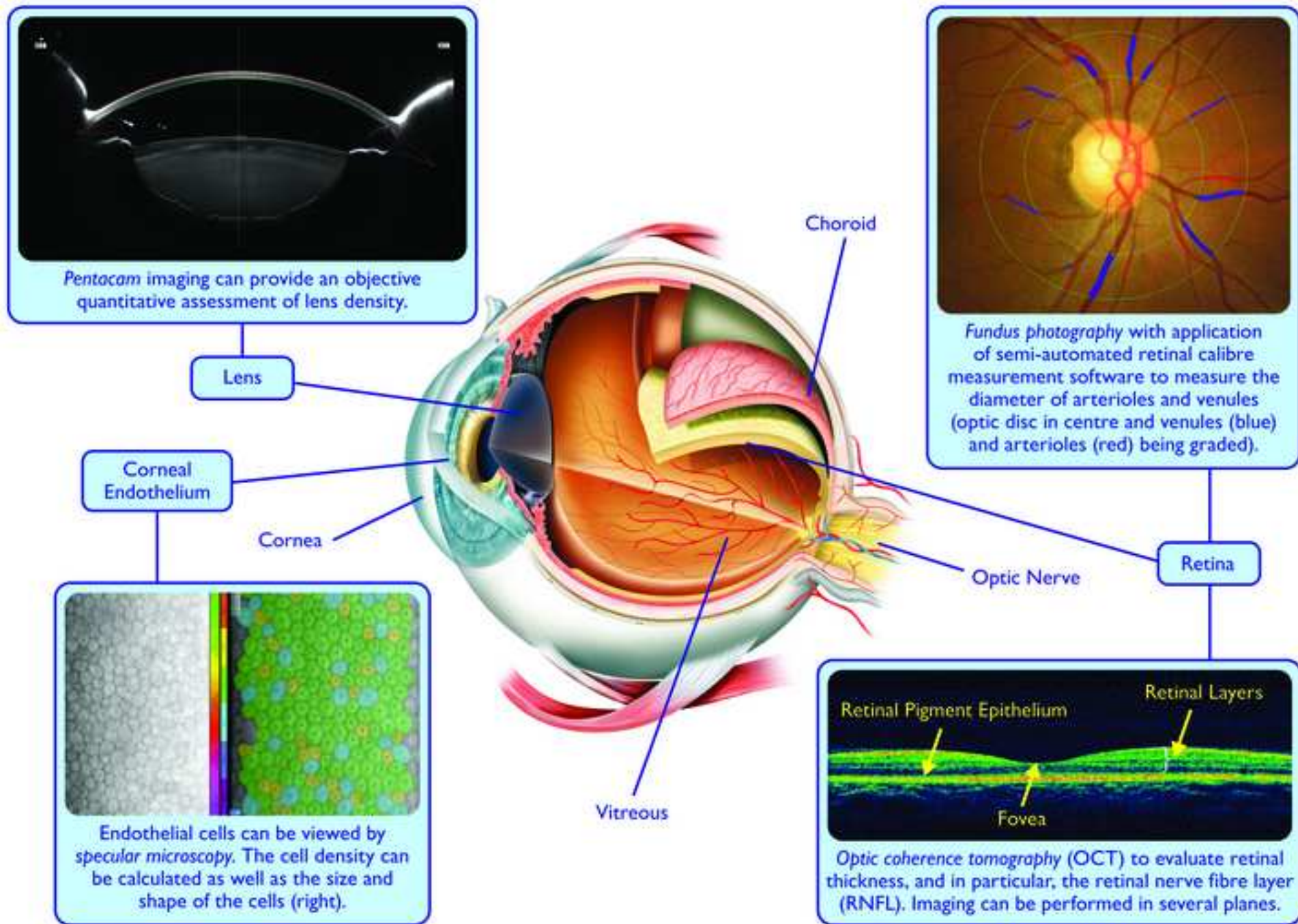
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Figure 1









Chapter 3

Research questions, hypotheses, study aims and objectives



Traffic intersection in Nyanga district of Cape Town with sign highlighting directions to the two study sites

**Linking material summarizing a statement of the problem,
research questions and hypotheses and
aims and objectives of the study**

3. I Statement of the problem

Increasing numbers of HIV-infected individuals are able to access HAART due to effective scale-up programmes in resource-constrained settings. This is likely to lead to an increased number of people living with HIV (See Figure 3c). Knowledge relating to accelerated aging in this group is important as the management of HIV may shift over time from primarily treatment of acute OIs to the management of age-related conditions in the long term. There are limited data/studies investigating accelerated aging and HIV in Africa, and the epidemiology of age-related diseases (as a function of increasing chronological age and accelerated aging) within the African HIV-infected population is poorly understood at present. Knowledge about the concept of accelerated aging in HIV may lead to the ability to predict it so that it can be modulated, or even prevented. Data on the burden and types of age-related conditions will assist in planning the best methods to screen for, prevent and manage these conditions. From a health policy perspective this information can also aid in anticipating the infrastructure and health systems needed to deliver care and treatment for individuals affected or at risk from these conditions.

Studies investigating accelerated aging in HIV have been conducted in the USA and Europe. However, these studies are likely to be confounded as the HIV-infected population in these locations are more likely to be exposed not only to risk factors known to accelerate aging (e.g. smoking and alcohol) but also to other exposures (e.g. recreational drugs, other 'lifestyle' factors) compared to their non-HIV-infected counterparts. The advantage of examining an HIV-infected cohort in a country such as South Africa (and comparing findings with a non-infected group) is that these two groups are more likely to be similar in terms of socio-demographic characteristics and risk factors, so reducing differential risk exposure. The NIH Office of AIDS Research has highlighted the need for carefully designed studies of HIV and aging that takes these factors into account [1].

Few studies have evaluated patients on HAART versus those who are treatment naïve – it may be that HIV itself is an accelerant of aging or that some of the immunosenescent effects are mediated by HAART. It may be difficult to assess the effect of HAART on accelerated aging. If HAART-naïve patients exhibit greater signs of aging (compared to treated patients) it would strongly suggest the likelihood of a (partially?) reversible process. Conversely, if HAART-treated patients show more signs of aging then this may suggest a cumulative, irreversible process. (Refer to Figure 3a for a pictorial representation of these hypotheses)

Examination of the eye in the context of HIV-related aging has not been undertaken to date. The eye may be a uniquely useful site as a model of aging. It is easily accessible and several of its components can be measured objectively and directly. The relationship between serum biomarkers of aging and clinical and/or ocular parameters of aging has also not been widely investigated. Understanding the changes in ocular parameters that occur with HIV may help to understand who is at risk of visual morbidity or blindness. Changes in eye (as a model of aging) may even be useful as a predictor of mortality.

3.2 Research questions

- Do HIV-infected individuals experience accelerated aging compared to age and gender-matched HIV-uninfected individuals in resource-constrained settings (where baseline CD4 counts are often low and viral loads high at HAART initiation)?
- Does the accelerated aging process also affect the ocular tissues (perhaps mediated by retinal para-inflammation) leading to visual loss or blindness? Can ocular parameters of aging predict who is at risk of visual loss?
- Are serum BoA levels raised in HIV-infected individuals demonstrated to have accelerated aging at the organ level? Do they correlate to ocular age-related parameters too?
- Does HAART affect accelerated aging? Or is HIV primarily responsible, with HAART exacerbating the process? Or does HAART ameliorate the process such that the aging trajectory is nearer to that of uninfected individuals? (See Figure 3b).

3.3 Study hypothesis, aims and objectives

Study hypotheses – HIV and accelerated aging:

The overall study hypothesis is that the processes of immunosenescence and accelerated aging, proposed to occur in HIV-infected individuals will be manifest in this population of South African HIV-infected adults. In particular:

- Age-related ocular parameters representing an ‘ocular aging’ phenotype will occur with greater frequency and severity in HIV-infected individuals compared with uninfected controls.
- Physical capability levels related to ‘frailty’ will be higher in HIV-infected individuals compared to non-infected counterparts.
- Serum biomarkers of aging (BoA) levels will be raised in HIV-infected individuals compared to age and gender-matched controls.
- Serum BoA levels will correlate with ocular and systemic measures of aging.
- HIV-infected individuals with ocular signs of aging are more likely to have a low nadir CD4 count and high viral load pre-ART.
- In HIV-infected individuals who are treatment-naïve, the ocular aging phenotype will be expressed at a higher frequency compared to HIV-uninfected individuals.
- HIV-infected individuals who are on HAART will express the ocular aging phenotype at a higher frequency compared to those who are treatment-naïve, suggesting that the change in ocular signs is a cumulative and irreversible process.

Figure 3a: Conceptual framework for study hypotheses

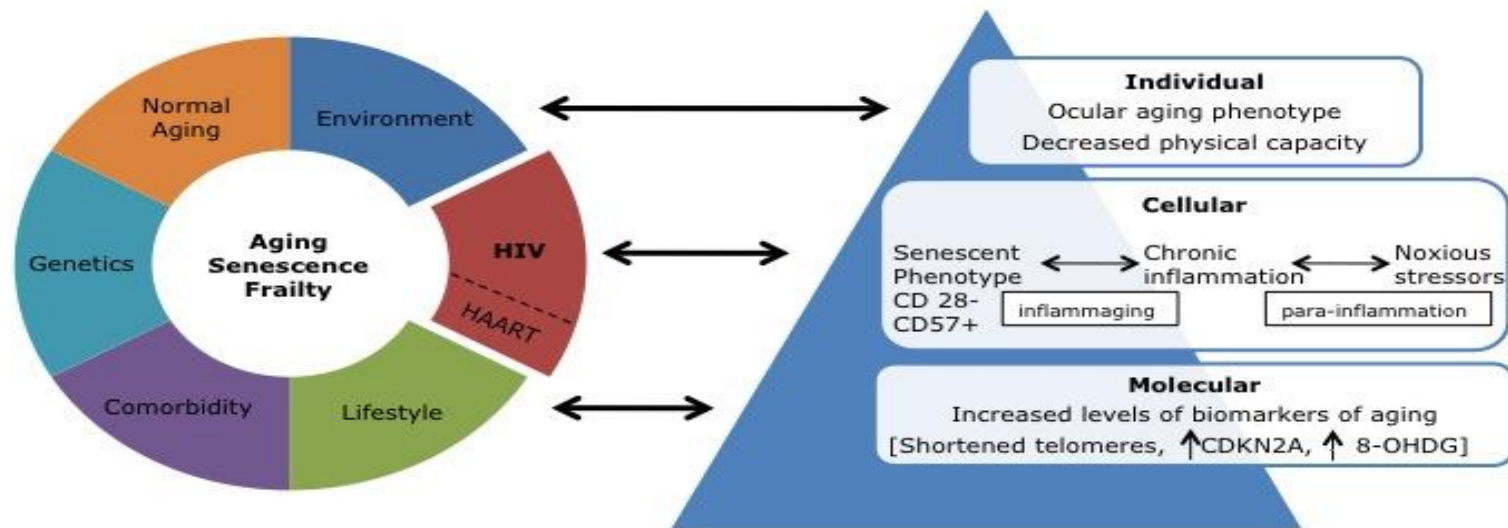
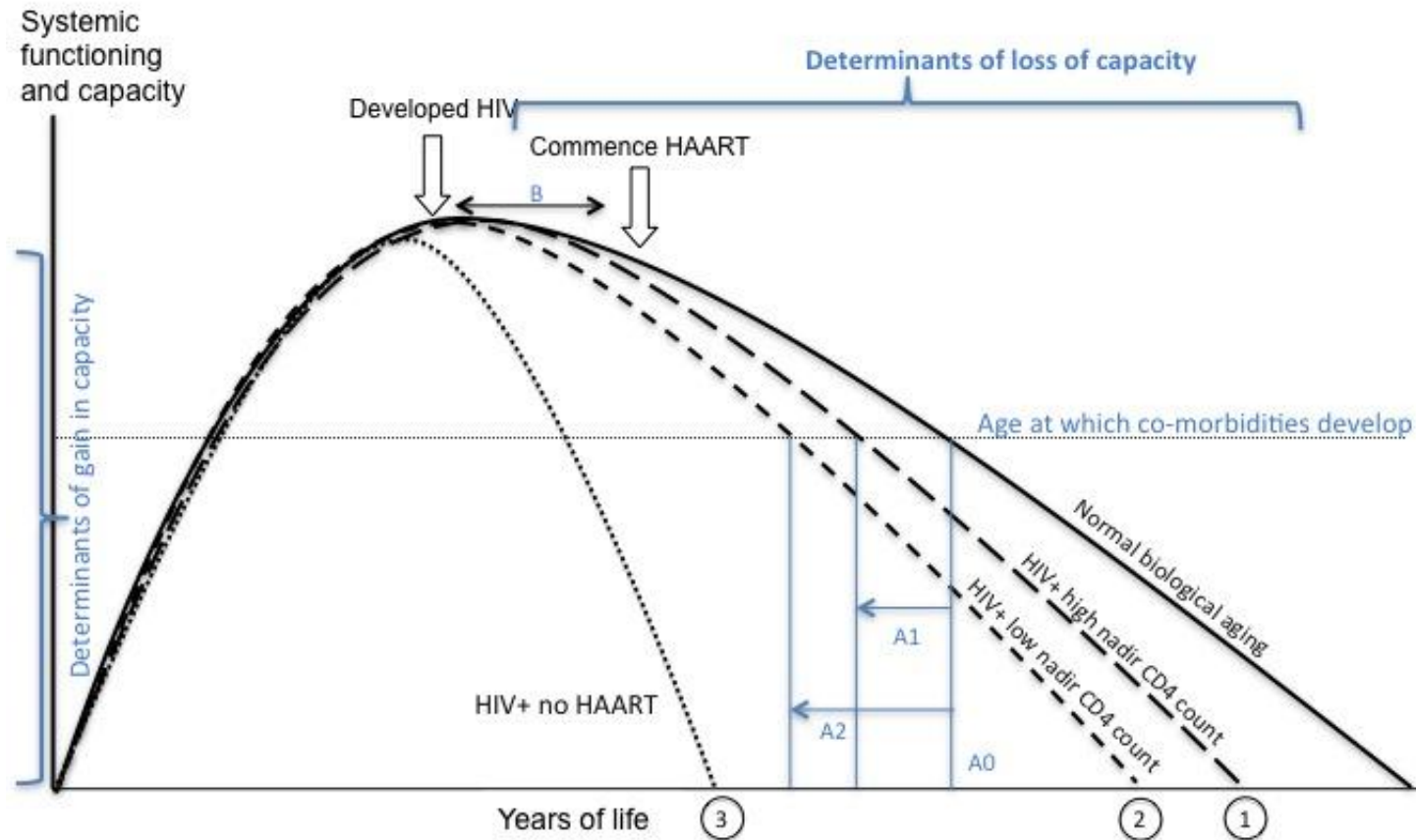


Figure 3b: Hypotheses relating to effect of HIV and HAART on accelerated aging – adapted from Kuh, 2007 [2]



Black line represents normal biological aging

1. HIV causes aging but HAART ameliorates it such that the ageing trajectory is nearer to what it would have been without HIV infection

2. HIV causes aging and low nadir CD4 count means that ageing trajectory does not reach potential compared to line 2.

3. Lifespan shortened due to death from opportunistic infections rather than related to accelerated biological ageing

B: Possible time frame when early HAART initiation may be able to influence ageing trajectory

A0: Chronological age at which co-morbidities develop in normal aging

Accelerated aging manifest as lowering of chronological age at which co-morbidities develop compared with those without HIV (A0): A1 HIV-infected individuals on HAART with high CD4 nadir count (>200 cells/ μ L) and A2 with low CD4 nadir count (<200 cells/ μ L)

3.4 Aim

To assess whether HIV-infected individuals demonstrate greater levels (frequency and severity) of ocular aging, systemic frailty and cellular senescence than a HIV-uninfected group of similar age.

Objectives:

1. To measure age-related parameters among a cohort of HIV-infected individuals in South Africa, including clinical evaluation of ophthalmic parameters and objective measures of physical capability.
2. To compare the measures of age-related parameters in the HIV-infected study population with a population of HIV-uninfected individuals who are gender- and age-matched.
3. To determine risk factors for ocular aging (among HIV-infected cohort) using *a priori* risk factors (e.g. nadir CD4 count; body mass index; history of TB; smoking; on HAART; duration on HAART).
4. To measure serum biomarkers of aging (telomere length, CDKN2A) and determine whether levels differ between the two study populations.
5. To determine whether clinical age-related parameters are correlated to serum biomarkers of aging.

3.5 Research Setting

3.5.1 Sub-Saharan Africa

In sub-Saharan Africa, 3 million people aged 50 years and older are living with HIV [3]. As ART scale-up continues, the prevalence of HIV in people over 50 years of age in South Africa will nearly double over the next 30 years [4]. Thus, understanding of the concepts of HIV and accelerating aging within the population has important healthcare and social implications.

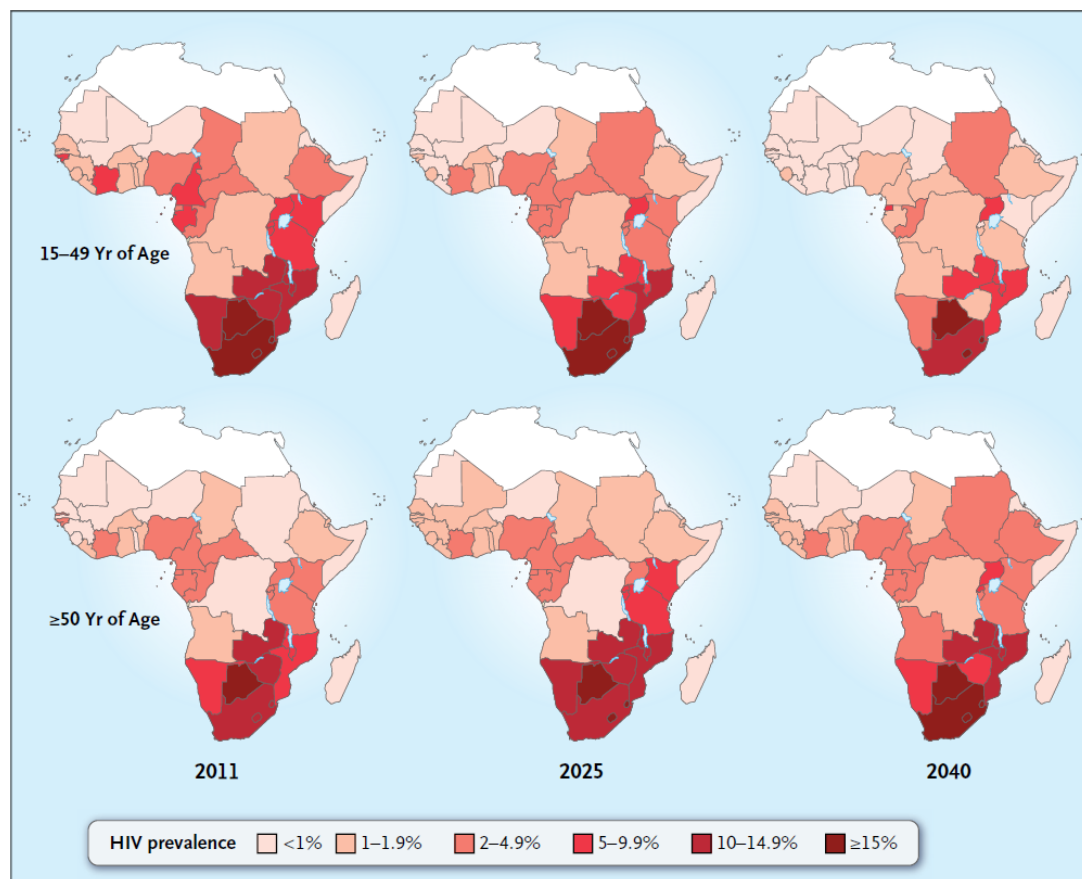


Figure 3c: HIV prevalence in Sub-Saharan Africa in 2011, 2025, and 2040 in the population 15 to 49 years of age and the population 50 years of age or older. Reproduced with permission from (Mills et al. *N Engl J Med* 366;14:1270-73), Copyright Massachusetts Medical Society.

3.5.2 Cape Town, South Africa

HIV epidemiology in South Africa:

Estimates from 2011 indicate 5.6 million (4.9-6.6 million) people are infected with HIV in South Africa (Figures 3d-f) [5]. Overall prevalence in adults is 18.1% (15.4-20.9%), but varies throughout the country, with some provinces more severely affected than others (Figure 3d). In 2005 the highest antenatal prevalence was in KwaZulu-Natal (39.1%) and lowest in the Western Cape (15.7%). Women account for approximately 55% of HIV-infected individuals. The prevalence in women aged 25-29 years is up to 40%. For men, the peak is at older ages, with prevalence estimated at 10% among men older than 50 years. HAART availability is being scaled up and recent estimates suggest 28-55% coverage for adults (Figure 3g). Eligibility to initiate HAART is according to the 2010 guidelines released by the South African National AIDS Council and the Department of Health i.e. CD4 count $<200\text{cells}/\text{mm}^3$ irrespective of clinical stage; CD4 count $<350\text{cells}/\text{mm}^3$ in patients with TB/HIV and/or Pregnant women; WHO stage IV irrespective of CD4 count; MDR/XDR-TB irrespective of CD4 count [6].

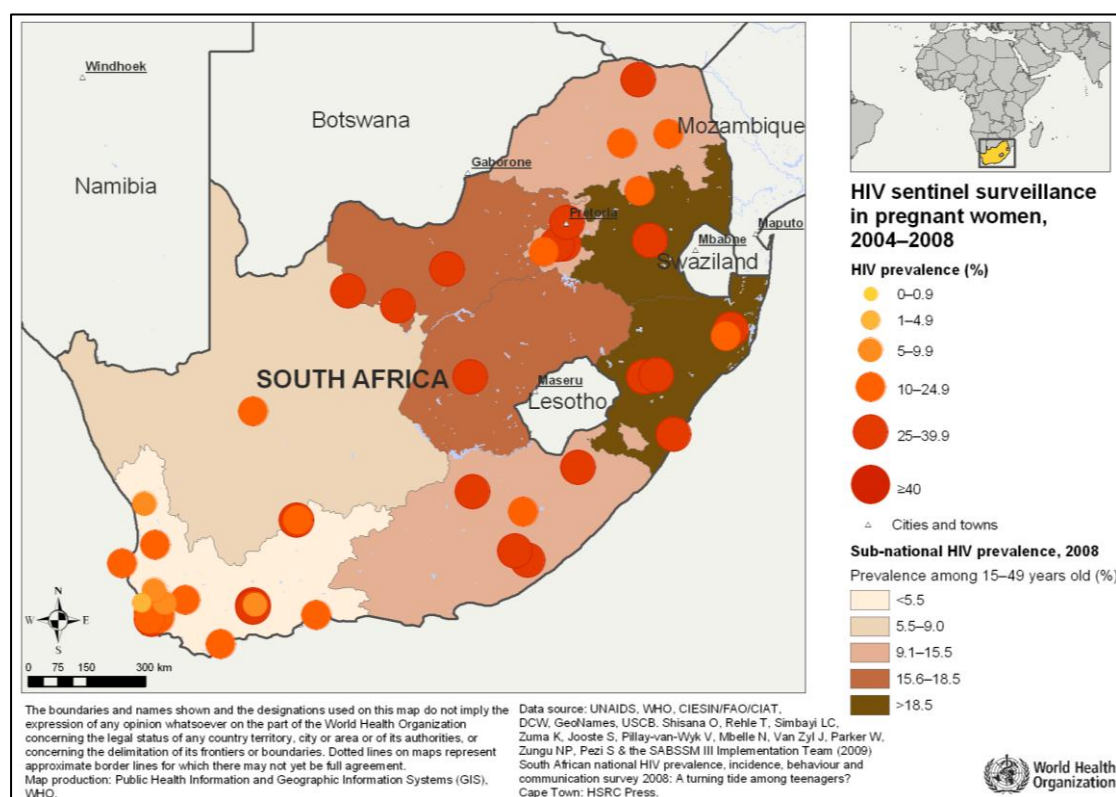


Figure 3d: Adult (aged 15-49 years) HIV prevalence in South Africa. Source UNAIDS, 2012

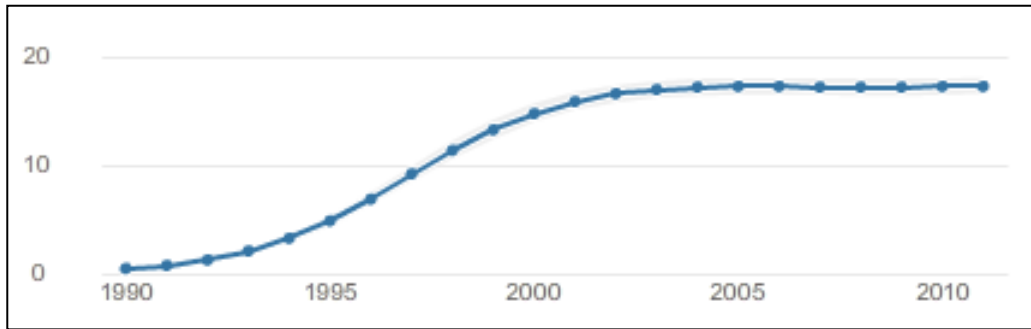


Figure 3e: HIV prevalence – Ages 15 to 49 years (%) in South Africa

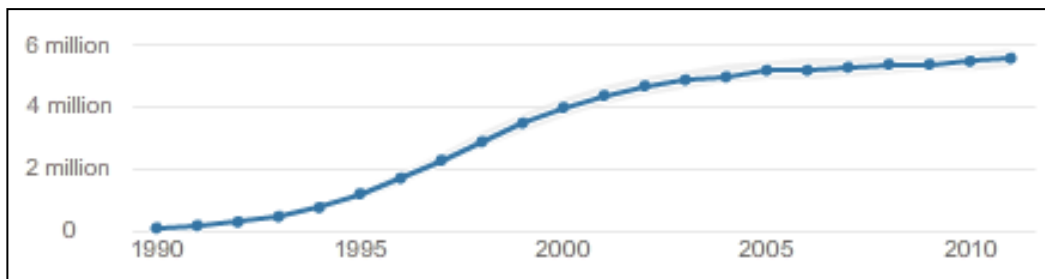


Figure 3f: Estimated number of people living with HIV in South Africa

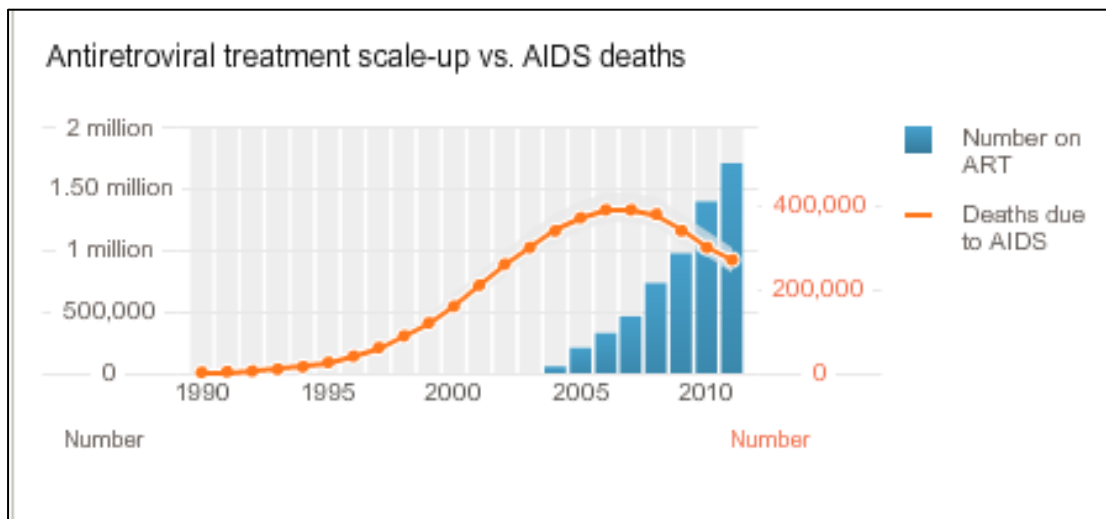


Figure 3g: Estimated number of people receiving ART vs. AIDS deaths

(Figures 3d-3g: Report on the global AIDS epidemic, 2012, UNAIDS; with permission)

Study sites: The Hannan Crusaid Treatment Centre is a joint project between the Desmond Tutu HIV Foundation and the Western Cape Department of Health. (Figure 3h). It is situated on the site of the Gugulethu Community Health Centre in the Gugulethu/Nyanga district, approximately 20km from the centre of Cape Town (see Map, Figure 3k). The clinic is a state of the art facility for HIV care in South Africa and has excellent levels of attendance and follow-up. This cohort of HIV-infected individuals is extremely well-characterised [7-12], and all clinical case records are updated into a computerised database, so detailed historical clinical and demographic information is easily accessible. The Department of Ophthalmology, Groote Schuur Hospital had space and facilities made available for this project.



Figure 3h: The Hannan Crusaid Treatment Centre, Gugulethu Township



Figure 3i: The Emavundleni Centre for HIV Research, Crossroads Township

Study populations: In 2005, the Gugulethu/Nyanga district HIV prevalence was 29.1%, the second highest in the province [13]. This is in contrast to the generally low prevalence in the Western Cape (Figure 3d). HIV vaccine trials are carried out at the Emavundleni Centre for HIV Research, also run by the Desmond Tutu HIV Foundation (Figure 3i). The centre is also located in the Gugulethu/Nyanga district. Community HIV education and awareness, free voluntary testing, counselling and basic health information is offered at this centre, and patients attending this centre are likely to have similar socio-demographics to those attending the Hannan Crusaid Treatment Centre. All individuals participating in vaccine trials had laboratory evidence of HIV-seronegative status. The district has basic amenities and consists of both formal and informal housing sectors. Many of the 'new' settlements surrounding Gugulethu have predominantly informal housing, with high levels of crowding and poverty (Figure 3j). The vast majority of the population are of African ancestry.



Figure 3j: Typical community housing and informal settlements at recruitment sites

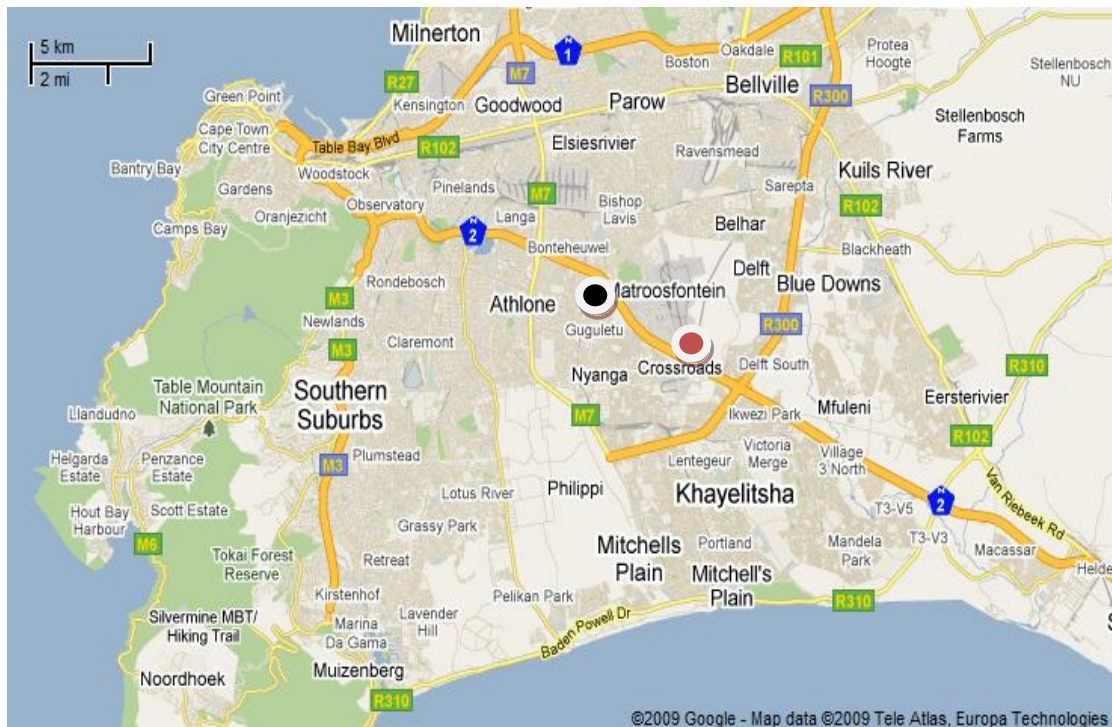


Figure 3k: Map of Cape Town showing relative positions of Gugulethu (black circle) and Crossroads (red circle) [From Google Maps]

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Chapter 4

Study protocol and description of data collection methods



Study clinical examination room, Dept. of Ophthalmology, Groot Schuur Hospital

**Linking material detailing the study protocol,
participant enumeration and examination techniques**

4.1 Pilot work undertaken to inform main study

Pilot study of age-related conditions in HIV-infected individuals in the Gugulethu HIV-infected study population

This was part of a pilot study to evaluate the prevalence of HIV-related eye disease within a cohort of HIV-infected individuals initiating HAART (*Pathai et al. [1]*)

In this study data were collected on all eye conditions (not just HIV-related) in HIV-infected individuals attending the Gugulethu clinic. Demographic and clinical data were obtained from clinic case records. Standardised ocular symptom screening was conducted relating to impairment of vision, pain and floaters/flashing lights.

Ophthalmic examination comprised visual acuity measurement, anterior segment examination (via portable slit lamp) and dilated indirect ophthalmoscopy. . Cataract was defined as any lens opacity regardless of impact on visual acuity. Maculopathy refers to presence of any pigmentary change at the macula. Functional presbyopia was defined as requiring at least +1 dioptre in order to read the N8 optotype (on a near visual acuity chart) at a distance of 40cm in the participant's usual visual state (with or without a spectacle correction for distance vision).

Data were available for 157 participants (314 eyes) of whom 65% were female. The median age was 34 years [interquartile range (IQR) =28-42). The median baseline CD4 count was 143 cells/ μ L (IQR=59-199), and viral load 37,328 copies/mL (IQR=8,936 -176,882). Past or current TB was present in 51% (n=80), and 62% (n=97) were staged at WHO clinical stages 3 and 4. Ophthalmic manifestations of HIV were detected in 12.1% (95%CI: 7.4-18.2; n=19) of the cohort. In those with CD4 counts <200 cells/ μ L (n=118), 13.6% (95%CI: 8.0-21.0, n=16) had HIV-related ocular conditions. The most common manifestation was HIV retinopathy, present in 4.5% (95%CI: 1.8-9.0; n=7) of the cohort. CMVR was seen in 2 cases (1.3%, 95%CI: 0.2-4.2).

Table 3.1 shows the distribution of the age-related conditions within the HIV-infected cohort. It is apparent that conditions typically associated with older age (cataract, maculopathy, presbyopia) are present before the age of 50 years in this population (note: presbyopia tends to occur at approximately 40 years of age). Similarly, these changes only become manifest in this population after the age of 30

years. Therefore, examination of participants aged 30 years and over from both study populations would be ideal for this study, and the proportion of individuals affected ≥ 30 years has been used to inform for sample size calculations (see Chapter 4, section 4.3). A limitation of this data is that outcomes were made solely on clinical examination, rather than using standardised grading systems, or with the use of photographic evidence (due to logistical and budgetary constraints).

Table 4.1: Prevalence of age-related conditions within HIV-infected study population* (Hannan Crusaid Clinic, Gugulethu) (total individuals sampled=157)

**All findings were based on clinical examination only and recorded as binary (absent/present) outcomes. Findings were recorded 'per person' rather than 'per eye' (if one eye had the condition this was considered presence of the condition at a person level, equally presence of the condition in both eyes was still recorded as affecting one individual)*

Condition	Age group								Overall n= 157
	0-29 n=49	30-35 n=41	36-40 n=23	41-45 n=18	46-50 n=12	51-55 n=7	56-60 n=5	>61 n=2	
Cataract	0% (0)	0% (0)	4.3% (1)	5.6% (1)	16.7% (2)	28.6% (2)	40.0% (2)	100.0% (2)	6.4% (10)
Maculopathy	0% (0)	4.9% (2)	4.3% (1)	0% (0)	16.7% (2)	14.3% (1)	0% (0)	0% (0)	3.8% (6)
Presbyopia	0% (0)	7.3% (3)	8.7% (2)	66.7% (12)	91.7% (11)	85.7% (6)	100% (5)	100% (2)	26.1% (41)

4.2 Study protocol

Study design: Case-control study of two clinic-based populations.

Setting: Nyanga/Gugulethu District, Cape Town (recruitment of participants).

Department of Ophthalmology, Groote Schuur Hospital, Cape Town (examination of participants).

Study population:

- i) **HIV-infected individuals:** Potential participants recruited from the Hannan Crusaid Treatment Centre, Gugulethu, Cape Town. Once a week the study coordinator recruited potential participants using the database held in the clinic.

Inclusion criteria:

1. Adults \geq 30 years.
2. Participant groups will be stratified into:
 - i. HAART-naive.
 - ii. On first-line HAART of known duration.
3. Give informed consent to undergo full ophthalmic examination, physical capability measures, and drawing of blood for serum biomarker analysis.

Exclusion criteria:

1. Adults $<$ 30 years.
2. Acute opportunistic infection (systemic or ocular).
3. Pre-existing ocular inflammatory disease (offered referral and further management as required).
4. Consent not given.
5. Known diabetic.

- ii) **HIV-uninfected individuals:** Potential participants were recruited from the Emavundleni Centre for HIV Research, Crossroads district, Cape Town. HIV vaccine trials are carried out at the Emavundleni Centre and participants had a negative HIV test within the preceding two months. These controls were frequency matched on gender and age using 5-year categories.

Inclusion criteria:

1. Adults \geq 30 years.
2. Give informed consent to undergo full ophthalmic examination, physical capability measures, and drawing of blood for serum biomarker analysis.

Exclusion criteria:

1. Adults <30 years.
2. Pre-existing ocular inflammatory disease (offered referral and further management as required).
3. Consent not given.
4. Known diabetic.

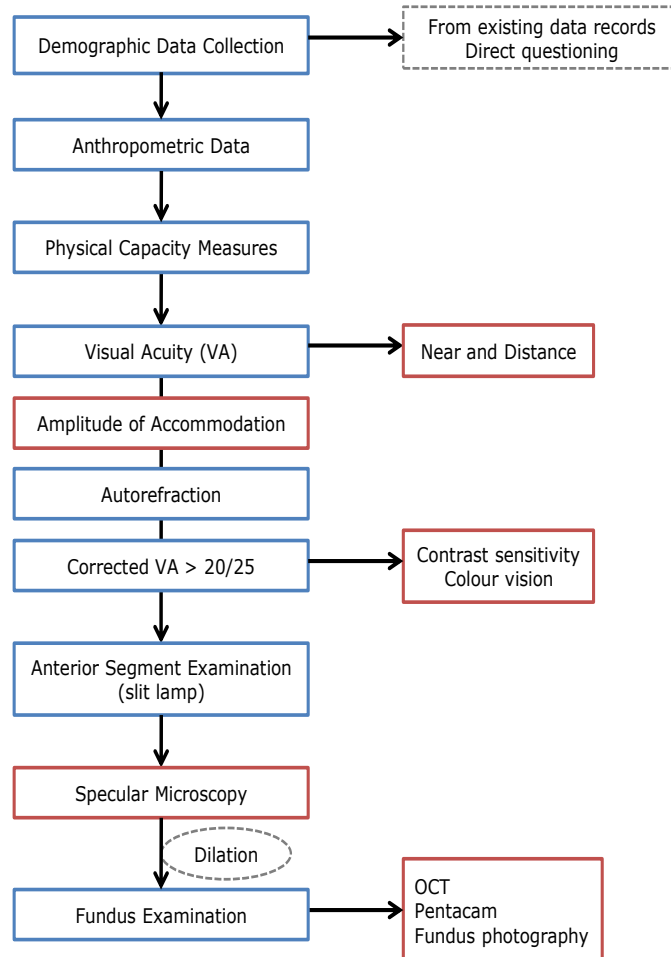
Ethics approval:

Approval obtained from: a) Ethics Committee, London School of Hygiene and Tropical Medicine, b) Ethics Committee, University of Cape Town Medical School. (See Appendix 1: Ethics Approvals). An Export Permit was obtained from the Government of South Africa to export blood samples to the UK for analysis.

Participant logistics:

Potential participants were informed about the nature of the study at both recruitment centres (Hannan Crusaid ART Clinic and Emavundleni Centre), and asked if they would be willing to undergo ophthalmic and physical examination. The study patient information document was either given for the potential participant to read or read to them in an appropriate language (Appendix 3: Patient Information Documents). Enrolment into the study was dependent on completion of a 'willingness to participate' form with a date and time to attend for assessment (Appendix 4). Participants were given partial remuneration to attend the examination site (Department of Ophthalmology, Groote Schuur Hospital) for formal assessment. At the examination site witnessed informed consent was recorded on a consent form marked by the participant's signature or thumbprint using forms in English/Xhosa after translation and back translation (Appendix 5: Consent forms). The participant then underwent assessment as detailed below (Figure 4a: Flowchart of participant evaluation).

Referral mechanisms and access to ophthalmic treatment: Eye/other conditions were managed accordingly by referral to the Dept. of Ophthalmology, Groote Schuur Hospital. Participants requiring presbyopic correction were given free spectacles. Treatment was provided free of charge.



Key:

Red-boxes Measurement of age-related ophthalmic parameters

Figure 4a: Flowchart of participant evaluation

4.3 Data collection and participant evaluation

Included data on the following (See Appendix 6: Data Collection form)

Demographic characteristics	HIV infected on HAART	HIV infected HAART-naïve	HIV uninfected
Socioeconomic data (to include smoking/alcohol)	X	X	X
Occupation: mainly indoor/mainly outdoor as proxy for UV exposure	X	X	X
Educational status	X	X	X
Medical history of e.g. hypertension, diabetes, cardiovascular disease	X	X	X
Ophthalmic history			
Family history of eye disease	X	X	X
Date first documented to have HIV infection and route of transmission	X	X	-
WHO clinical staging of AIDS-defining illness	X	X	-
Tuberculosis status	X	X	X
Nadir and current CD4+ count	X	X	-
Highest/most recent HIV viral load (HIV RNA levels)	X	X	-
HAART treatment history (when commenced, regimen)	X	-	-
History/number of opportunistic infections	X	X	-
Clinical/anthropometric data			
Height (metres)	X	X	X
Weight (kg)	X	X	X
Blood pressure	X	X	X
Fasting blood glucose	X	X	X

Data were collected in particular relating to exposures known to affect aging and age-related parameters (e.g. smoking and UV exposure both risk factors for aging and lens opacities). Changes in practice at the clinic meant that not all HAART-naïve participants had a HIV RNA level taken pre-treatment.



Figure 4b: Participant interview in preferred language

Participant examination (refer to flowchart, Figure 4a)

I. Ophthalmic examination – full ophthalmic examination by PI and study coordinator



Figure 4c: Slit lamp examination by ophthalmologist

2. Measurement of age-related ocular parameters:

(Refer to Chapter 2 'The eye as a model for aging' for further information)

Parameter	Method of measurement	Measurement obtained
Distance/near visual acuity	ETDRS visual acuity chart	No of lines/letters read
Amplitude of accommodation	RAF rule	Dioptric value
Contrast sensitivity	Pelli-Robson chart	CS score
Colour vision	Ishihara plates	Total score
Corneal endothelial cell count	Specular microscopy	Endothelial cell count
Lens opacity (cataract)	Pentacam with lens densitometry software	Lens density value
Retinal nerve fibre layer (RNFL)	Optical coherence tomography (OCT)	Thickness in μm
Retinal vessel diameter	Fundus photography then IVAN retinal vessel analysis software	Thickness in μm



Figure 4d: Assessment of visual acuity using LogMAR chart



Figure 4e: Assessment of contrast sensitivity using Pelli-Robson chart



Figure 4f: Corneal endothelium assessment using specular microscope



Figure 4g: Pentacam acquisition (typically acquired in darkened environment, taken in light for illustrative purposes)

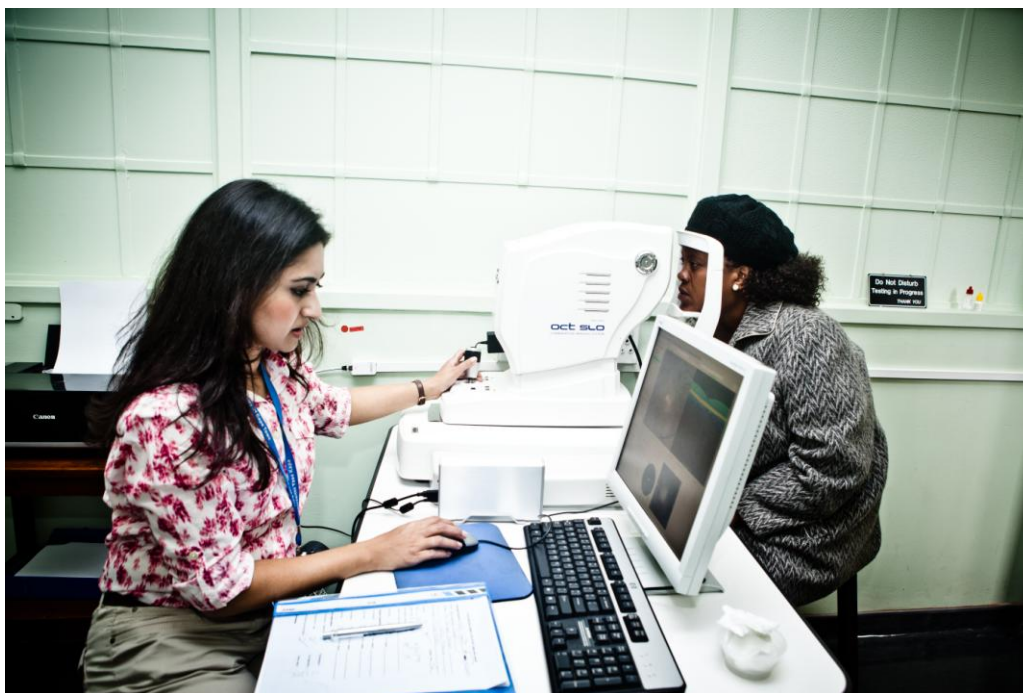


Figure 4h: Measurement of retinal nerve fibre layer (RNFL) using optical coherence tomography (OCT)



Figure 4i: Fundus photography to obtain images for retinal vessel calibre assessment

3. Objective measures of physical capability.

1. Grip strength – with grip dynamometers
2. Walking speed – over 6 metres
3. Chair rising – from a standard chair, with or without arms
4. Standing balance – over a 6 metre long narrow path



Figure 4j: Example of participant undertaking chair rises as part of physical capability assessment

4. Evaluation of serum biomarkers

Blood samples were taken for DNA/RNA and plasma extraction, and subsequent evaluation of levels of BoAs (telomere length, CDKN2A, 8-OHDG). Samples were taken from all participants excluding the first 79 participants due to difficulties in obtaining appropriate consumables at initiation of the study. Refer to Chapter 5 for details of sample extraction.



Figure 4k: DNA extraction machine (Maxwell™ Automated Purification System, Promega, USA). Whole blood mixed with Trizol reagent (Invitrogen, UK) for RNA extraction

Following extraction, all samples were transferred to a deep freezer and stored at minus 80°C and then shipped to the University of Glasgow on dry ice.

4.4 Power and sample size calculations

4.4.1 Need for pilot data collection from both study populations

To obtain an approximation of the sample size required for this study, reference to published data was made. Where possible, estimates were derived from epidemiological population-based studies in African populations or from studies where the proposed ocular parameters have been measured in an HIV-positive population.

Outcome measures

The main outcome measures related to presence of lens opacity, change in RNFL, amplitude of accommodation, and change in retinal vessel diameter. Secondary outcome measures included measures of contrast sensitivity, colour vision, endothelial cell count, physical capability measures and levels of serum biomarkers.

Sample size calculations – using normative data

Normative data for age-related eye conditions is generally limited to populations over the age of 40-50 years (as these conditions typically affect the older age groups). Data on cataract prevalence were obtained from a large population-based study conducted in Nigeria [2]. Data on RNFL was obtained from publications comparing this parameter between HIV-infected and non-infected individuals [3,4]. For assessment of retinal vessel calibre and amplitude of accommodation, publications were reviewed that compared these parameters in different age groups [5-7] (Tables 4.2 and 4.3).

Table 4.2: Estimated sample size for two-sample comparison of proportions (assuming equal numbers of HIV infected and HIV uninfected individuals).

Outcome	Proportion in HIV+ population (≥30 years) ^{\$}	Proportion in HIV – population (≥30 years)	Odds ratio detected	Significance level (%)	Power (%)	Required HIV infected	Required HIV uninfected
Cataract	9.3%	3.5% [#]	2.8	95	90	406	406
				95	80	312	312

^{\$} - refers to HIV-infected individuals attending Hannan Crusaid Clinic, Gugulethu

[#] - crude prevalence of cataract with presenting vision <6/12-6/60

Table 4.3: Estimated sample size for two-sample comparison of means.

Outcome	Significance level	Power (%)	Number required HIV infected	Number required HIV-uninfected	Difference detected*
RNFL	95	90	52	52	10% difference in retinal thickness, assuming SD of 15.0µm
	95	80	40	40	
Retinal vessel diameter	95	90	141	141	5% difference in diameter, assuming SD of 21.0µm
	95	80	108	108	
Amplitude of accommodation	95	90	60	60	15% difference in AA assuming SD of 0.5 diopres
	95	80	45	45	

*Difference reported as statistically significant in published data

4.4.2 Feasibility and logistics

The sample size calculations indicate that a sample size of 640 (320 HIV infected and HIV uninfected) would have at least 80% power to detect a statistically significant difference for all parameters listed (in terms of the given proportions and means). This sample size was thought to be practically and logistically feasible at the proposed study site based on the discussion with local coordinators and staff. In practice, the sample size was smaller – refer to following section.

The power achieved for the different parameters with 640 participants is listed in Table 4.3. In summary, a sample size of 320 cases and 320 controls will be sufficient to detect the differences outlined in Tables 4.2 and 4.3, at a 95% significance level, and greater than 80% power for the outcome variables listed (Table 4.4).

Table 4.4: Power for study assuming 95% significance level

Outcome	Power with this sample size (%)	
	HIV infected/uninfected: 320/320	HIV infected/uninfected: 160/320**
Cataract	81	60
Retinal diameter	99	97
RNFL	99	99
Amplitude accommodation	99	99

** Secondary analyses initially planned with stratification of the HIV-infected group into those on HAART and those who are HAART-naive. If half of HIV-infected group are HAART-naive, then analysis of this group (n=160) with the HIV-uninfected cohort (n=320) will still have sufficient power to detect a difference for the majority of outcomes

4.4.3 Actual power with sample size obtained:

The actual number of participants recruited during the data collection period was 504, composed of 248 HIV-infected individuals and 256 HIV-seronegative participants.

Table 4.5 illustrates the power obtained with this sample size (assuming 1:1 ratio of 252:252 HIV+/HIV-).

Table 4.5: Power achieved with actual sample size obtained assuming 95% significance level

Outcome	Difference detected	Power (%)
Cataract	Difference in cataract prevalence as Table 4.1	70%
Retinal diameter	10% difference in retinal thickness, assuming SD of 15.0 μ m	98%
RNFL	5% difference in diameter, assuming SD of 21.0 μ m	98%
Amplitude of accommodation	15% difference in AA assuming SD of 0.5 dioptres	99%

The table indicates that good power was still achieved despite the reduced sample size obtained for the study.

Data management:

Dedicated Access databases were made for all the data, with range and consistency checks. All data was double entered. Data forms were kept in secure cabinets, data in the database was anonymized and databases password protected. Patient identifiers were stored as two separate databases, so that an individual's unique information was not accessible from just one database.

Analytical approach for ophthalmic data

Murdoch *et al* [8] have outlined the methods of analysis that can be undertaken for clinical ophthalmic studies.

1. The level of the individual –where both eyes are required for a diagnosis (e.g. blindness/visual impairment)

2. One eye per individual to be used in the analysis – one eye is chosen at random or ‘right only’ or ‘left only’ – can be used in studies of risk factors that act at the individual level.

3. Overall summary of ocular findings per individual – pooled data from both eyes, and the average taken.

4. Analysis at ocular level – to overcome correlation between eyes random effects models can be used [9,10], or generalised estimating equations [11]. This correlation between observations must be taken into account in the statistical analyses; if not there are likely to be errors in the result such as falsely precise confidence intervals.

As parameters will be related to the HIV status of the individual, data were analysed at the individual level. One eye per individual was used in the analysis, chosen at random. However not all eyes were suitable for measurement, for example eyes that have had significant cataract were unlikely to optimal RNFL or vessel calibre images, and so these measures would not be valid. In this case the contralateral eye will be chosen for measurement of eye parameters.

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Chapter 5

Accelerated biological aging in HIV-infected individuals in South Africa: a case-control study



Children playing on a street in Crossroads Township, Cape Town

Research paper comparing cellular biomarkers of aging (telomere length and CDKN2A expression) in HIV-infected and HIV-uninfected individuals

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work
2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **PLOS Medicine**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, SD Lawn, CE Gilbert, D McGuinness, L McGlynn, HA Weiss, J Port, T Christ, K Barclay, R Wood, LG Bekker, PG Shiels
 - 2.3. Stage of publication – ~~Not yet submitted/~~**Submitted/**~~Undergoing revision from peer reviewers' comments/~~In press
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments with assistance from Paul Shiels and Clare Gilbert. I conducted the data collection with assistance from the research study nurse. I performed DNA extraction, quantification and RNA preparation on site in Cape Town. I performed the statistical analyses with advice from Helen Weiss. Paul Shiels' laboratory team were responsible for measurement of CDKN2A levels and telomere length. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



Accelerated biological aging in HIV-infected individuals in South Africa:

A case-control study

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Abstract

Background: Little is known about the impact of HIV infection on biological aging in sub-Saharan Africa. We assessed two validated biomarkers of aging, telomere length and CDKN2A expression (a mediator of cellular senescence), in South African HIV-infected adults and HIV-seronegative individuals.

Methods: Case-control study of 236 HIV-infected adults aged ≥ 30 years and 250 age- and gender frequency-matched HIV-seronegative individuals. Biological aging was evaluated by measurement of telomere length and CDKN2A expression in peripheral blood leukocytes.

Results: The median ages of the HIV-infected and HIV-seronegative participants were 39 years and 40 years respectively. Among HIV-infected participants, 87.1% were receiving anti-retroviral therapy (ART), their median CD4 count was 468 cells/ μ L and 84.3% had undetectable viral load. Both biomarkers were validated against chronological age in HIV-seronegative individuals. Telomere length was significantly shorter in HIV-infected individuals compared to HIV-seronegative individuals (mean relative T/S ratio \pm SE: 0.91 ± 0.007 vs. 1.07 ± 0.008 , $p < 0.0001$). CDKN2A expression was higher in HIV-infected participants compared to HIV-seronegative individuals (mean expression: 0.45 ± 0.02 vs. 0.36 ± 0.03 , $p = 0.003$). In participants on ART with undetectable viral load, biomarker levels indicated greater biological aging in those with lower current CD4 counts. Lower income was associated with increased CDKN2A expression in HIV-seronegative individuals, but socio-economic factors were not associated with biological aging in HIV-infected participants.

Conclusions:

Telomere length and CDKN2A expression both indicated increased biological aging in HIV-infected individuals compared to HIV-seronegative individuals. These findings have potentially important implications for age-related morbidity among the millions of patients receiving ART in Africa.

Abstract word count: 237

Keywords: HIV; telomeres; CDKN2A; accelerated aging; biomarkers of aging; Africa

Suggested running head: Accelerated biological aging in HIV infection

MAIN TEXT

Background

HIV-infected individuals are at increased risk of age-related non-AIDS morbidity and mortality compared with HIV-uninfected persons [1]. It is speculated that HIV-infected individuals may not only be aging chronologically, but also undergoing accelerated biological aging mediated by increased cellular senescence [2].

Chronological age is an imprecise measure of biological aging, due to inter-individual differences in rates of aging. The disconnection between chronological age and lifespan has led to a search for effective and validated biomarkers of aging (BoA), defined as “biological parameters of an organism that either alone or in some multivariate composite will better predict functional capability at some late age, than will chronological age” [3].

Telomeric DNA length is a widely used BoA. Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. Their DNA component shortens with somatic cell division and upon reaching a critically short length, a DNA damage signal leads to cell cycle arrest, resulting in replicative senescence [4]. Telomere shortening is associated with increasing chronological age, and a wide range of pathologies, including cardiovascular disease [5] and renal dysfunction [6]. Telomere attrition is affected by psychosocial confounders, genetics and potentially by nucleoside reverse transcriptase inhibitors (NRTIs) [7]. Expression levels of the cell cycle regulator CDKN2A may represent a more robust BoA [8]. CDKN2A acts as a tumour suppressor and maintains cells in a state of growth arrest both in replicative and stress induced premature senescence. Increasing levels of CDKN2A transcriptional expression occur with increasing age both in solid organs and peripheral blood leucocytes (PBLs) [9]. In the former, increasing CDKN2A expression correlates directly with decreasing function.

Data from industrialized countries on the impact of HIV on accelerated aging may be confounded by differential risk exposure by HIV status to risk factors, such as smoking and alcohol consumption. Moreover, lower socio-economic status and poor diet are also associated with accelerated biological aging [10]. Thus, the NIH Office of AIDS Research has highlighted the need for carefully designed studies of HIV and aging that takes these factors into account [11]. Few studies have evaluated biological aging in resource-limited settings, where the AIDS epidemic is most severe. In sub-

Saharan Africa, 3 million people aged 50 years and older are living with HIV [12], and as ART scale-up continues, the prevalence of HIV in people over 50 years of age in South Africa will nearly double over the next 30 years [13]. If premature biological aging is associated with HIV, then age-related morbidity in HIV-infected individuals is likely to place a significant burden on healthcare systems in sub-Saharan Africa. The aim of this study was to test the hypothesis that HIV-infected individuals display advanced biological aging by directly comparing telomere length and CDKN2A expression in South African HIV-infected and HIV-seronegative individuals.

Methods

Ethics statement

The study was approved by the Ethics Committees of the London School of Hygiene and Tropical Medicine and the University of Cape Town Faculty of Health Sciences, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study participants:

HIV-infected individuals aged ≥ 30 years were enrolled from a community-based HIV treatment centre in Nyanga district in Cape Town [14]. All participants had a confirmed serological diagnosis of HIV and either about to commence ART (ART-naïve) or were already on first-line ART. HIV-seronegative participants were recruited using frequency-matching by gender and 5-year age categories. HIV-seronegative individuals were enrolled from participants confirmed to be HIV-seronegative attending an HIV prevention trials centre (Emavundleni Centre). These two centres were chosen, as attendees were drawn from the same community and therefore likely to have similar socio-demographic characteristics.

Data and sample collection

Socio-demographic information and medical history were obtained by questioning participants in their first language (Xhosa or English). Data collected included factors known to affect aging (e.g. indoor/outdoor occupation as a proxy for UV exposure). Clinical information was available relating to current and nadir CD4 counts, peak and current HIV plasma viral load (VL) and WHO clinical status. Venous blood was

collected at the time of participant interview to measure telomere length and CDKN2A expression in PBL.

DNA/RNA extraction

DNA was extracted from PBLs using the Maxwell™ Automated Purification System according to manufacturer's instructions (Promega, USA). DNA concentration and purity was quantified by Nanodrop Spectrophotometer (ThermoFisher Scientific, USA). RNA was extracted using Trizol reagent (Invitrogen, UK) following manufacturer's guidelines. DNA/RNA extraction was performed in Cape Town and samples shipped on dry ice to the University of Glasgow.

Telomere length determination

Telomere lengths were determined by QPCR following the method of Cawthon [15]. Telomere length determination was performed blindly using a Roche Light Cycler LC480. Briefly, telomere length analyses were performed in triplicate for each sample, using a single-copy gene amplicon primer set (acidic ribosomal phosphoprotein, 36B4) and a telomere-specific amplicon primer set [16]. Quality control parameters for the amplifications comprised a cut off 0.15 for the standard deviation (SD) of the threshold cycle (Ct) for sample replicates. At a SD above 0.15 the sample was reanalysed. The average SD across plates was 0.05. Relative telomere length was estimated from Ct scores using the comparative Ct method after confirming that telomere and control gene assays yielded similar amplification efficiencies. This method determines the ratio of telomere repeat copy number to single copy gene number (T/S) ratio in experimental samples relative to a control sample DNA. This normalised T/S ratio was used as the estimate of relative telomere length (Relative T/S). The inter-assay variation was assessed by comparing the relative telomere estimates (T/S ratio) estimates across assays for the positive controls, assayed on every assay plate. The average inter-assay coefficient of variance was 0.6% for telomere length and 0.23% for 36B4.

CDKN2A expression determination

Relative quantitative real-time PCR (qRT-PCR) was used to estimate mRNA levels corresponding to the candidate senescence associated gene - CDKN2A. Expression levels were measured against a reference hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene on an ABI Prism(R) 7500 Sequence Detection System.

Sequences of human TaqMan™ Primer/Probe sets were designed by Primer Express algorithm (Applied Biosystems, Austin, TX, USA). The comparative threshold cycle method ($\Delta\Delta CT$)[17] was employed to quantify relative gene expression.

Statistical Analysis

Analyses were performed using Stata 12 (Stata Corp, USA). Clinical and biochemical data were summarized as the median with interquartile range (IQR) or mean with standard error (SE), as appropriate. Analyses were conducted on log₁₀-transformed values of telomere length and mean CDKN2A expression to satisfy the assumption of normally distributed residuals. Results are displayed back-transformed to the original scale. Univariable analyses were performed to assess the relationships between mean telomere length, CDKN2A expression, HIV status and other clinical/demographic categories. Multivariable linear regression was used to examine the relationships of biomarker expression with HIV status adjusting for confounders identified in the univariable analysis and for *a priori* defined confounders (age and gender).

Results

Participant characteristics

Characteristics of the 236 HIV-infected individuals and 250 age/gender frequency matched HIV-seronegative individuals are reported in Table 1. All participants were of African ancestry. Telomere data were available for all participants, and CDKN2A data for 444 participants (91.4%). The majority (75%) of the study population was female. The median age of the HIV-infected population was 39 years (IQR 35-46 years), similar to the HIV-seronegative group (median 40 years (IQR 35-49 years) ($p=0.17$)). HIV-infected participants tended to be of higher socio-economic status, and were less likely to smoke or consume alcohol (Table 1). Cases also had a lower mean BMI and were more likely to have current or previous TB than HIV-seronegative individuals. Overall, 87.1% of HIV-infected participants were receiving ART and the current CD4 count among these participants was 468 cells/ μ L (IQR: 325-607 cells/ μ L) and 84.3% had undetectable VL (<50 copies/mL). All participants on ART received a regimen that contained an NRTI.

Biological age and chronological age in HIV-seronegative individuals

Telomere length and CDKN2A levels were validated against chronological age in HIV-seronegative individuals. As expected, there was a negative association between chronological age and telomere length (Pearson $r=-0.13$, $p=0.05$), and a positive association with CDKN2A expression ($r=0.16$, $p=0.02$). The relationship between CDKN2A expression and age was similar in males and females. However, for telomere length, age-related attrition was somewhat greater in males than in females but this difference did not reach statistical significance ($r=-0.25$ vs. $r=-0.09$, p -interaction= 0.13).

Biological age and HIV status

Telomere length was significantly shorter in HIV-infected individuals compared to HIV-seronegative individuals (mean relative T/S ratio [Rel T/S] \pm SE: 0.91 ± 0.007 vs. 1.07 ± 0.008 , $p<0.0001$, Figure 1a). Telomere length decreased with chronological age in HIV-infected individuals ($r=-0.15$, $p=0.03$). Mean CDKN2A expression was higher in HIV-infected participants compared to HIV-seronegative individuals (0.45 ± 0.02 vs. 0.36 ± 0.03 , $p=0.003$, Figure 1b, Table 2), and there was little evidence of correlation between chronological age and CDKN2A expression in HIV-infected individuals ($r=0.09$, $p=0.17$). No interactions were detected with HIV status when assessing the relationship between biomarkers and chronological age (data not shown).

Among the HIV-infected patients, there was no evidence that either telomere length or CDKN2A expression were associated with ART status ($p=0.71$ telomere length; $p=0.80$ CDKN2A; Table 2).

Among the 172 participants on ART with viral suppression, current CD4 count was positively associated with telomere length and negatively associated with CDKN2A expression (p -trend= 0.02 telomeres; p -trend= 0.05 CDKN2A; Table 3). There was no evidence of an association between these BoA and CD4 count in patients with detectable VL (data not shown).

Biological age and socio-demographic characteristics

Since socio-demographic characteristics may be associated with biological aging [10,18], we analyzed their association with the two BoA, stratified by HIV status (Tables 4a&b). HIV-infected participants aged ≥ 50 years had significantly shorter

telomeres than HIV-seronegative individuals in the same age group (mean RelT/S 0.84 vs. 1.01, $p=0.03$; Table 4a). There were no significant associations between telomere length and other socio-demographic variables for HIV-infected participants. In HIV-seronegative individuals, an association with alcohol consumption and telomere length was detected, with those who did not consume alcohol and those who consumed $>1L$ /week having the shortest telomere lengths ($p=0.04$) (Table 4a). For CDKN2A expression, the only evidence of an association was among HIV-seronegative individuals, where CDKN2A expression was higher among those with lower incomes than higher incomes (mean RelT/S 0.39 vs. 0.30, $p=0.03$; Table 4b).

Discussion

Two validated biomarkers of aging (telomere length and CDKN2A expression) were found to be consistent with increased biological aging in South African HIV-infected individuals compared with age- and gender-matched HIV-seronegative controls. This important finding is supported by our previous observations in this same study population that HIV infection is associated with increased frailty [19], as well as changes in retinal vessel calibre that are consistent with aging and increased cardiovascular risk [20]. Among patients receiving ART and in whom plasma viral load was suppressed, the biological aging estimated by both biomarkers was greatest in those with low current CD4 counts. These findings have potentially important implications for age-related morbidity among the millions of patients receiving ART in Africa.

Both biomarkers were strongly associated with chronological age in HIV-seronegative individuals, validating their use as BoAs. Reduced telomere length is associated with markers of low socio-economic status in industrialized countries [10,18]. Lower income was associated with increased CDKN2A expression in HIV-seronegative individuals, but socio-economic factors were not associated with either BoA in HIV-infected participants. Adjustment for socio-economic factors did not alter estimates of BoA between HIV-infected participants and HIV-seronegative individuals, suggesting that HIV infection, rather than social deprivation, is the main driver of biological aging in this population.

Aging reflects an accumulation of multiple molecular deficits in varying organ systems [21]. Inter-individual differences in rates of aging have prompted the search

for informative biomarkers of biological aging. Measurement of telomere length in PBLs is the standard method of evaluating biological aging, with changes in PBL telomere length in effective synchrony with changes in telomere length in solid organs, thus providing a suitable surrogate for biological age in the whole organism [22]. However, there is potential for confounding by methodological and design difference between studies [8]. The number of techniques available for telomere length measurement highlight that no one technique is entirely satisfactory. Southern blotting of terminal restriction fragments, single telomere length analysis (STELA) and real-time quantitative PCR (qPCR) may also be used to assess telomere length in PBLs. We chose to use qPCR, as it is the most suitable methodology for high throughput analyses [8,23]. Although inter-assay comparisons are very good (as evidenced by our CV of 0.6%), intra-laboratory comparisons can be poor [24]. In view of these methodological issues, we elected to measure an additional BoA, CDKN2A. CDKN2A expression increases with increasing chronological age in PBLs and solid organs [9,16,25], and it functions as a direct marker of cellular growth arrest [9]. CDKN2A represents a superior BoA to telomere length when judged by the Baker and Sprott criterion [3]. It can be a stronger predictor of function than chronological age and displays less inter-individual variation [16,25].

CDKN2A expression was substantially greater in HIV-infected adults in comparison to age-matched HIV-seronegative individuals. As well as functioning as a tumour suppressor, CDKN2A is also a component of stress-induced premature senescence [26] which prevents T-cell replication following acute insult [27]. The decoupling of the relationship between CDKN2A expression and chronological age in HIV infected individuals is a direct consequence of HIV-associated premature T-cell senescence [2], and lack of further T cell replication with HIV infection. Nelson et al [28] found that ART-naïve individuals had higher levels of CDKN2A expression compared to HIV-seronegative individuals, and suggested that active HIV replication accounted for elevated expression. Our data show that with viral suppression and ART, high levels of CDKN2A are still detectable. Thus, high rates of HIV replication are not a pre-requisite for continued, elevated expression of this biomarker.

Both biomarkers were significantly associated with current CD4 count in those receiving ART in whom viral load was undetectable. Increased CDKN2A expression was associated with lower CD4 counts, consistent with the finding that

CDKN2A expression is inversely related to T-cell replicative capacity [27]. Telomere length was also shorter in participants on ART with lower current CD4 counts. Thus, levels of both BoA suggested that lower current CD4 cell counts were associated with greater biological aging. Human telomerase comprises a reverse transcriptase sharing homology with the HIV reverse transcriptase [29]. It is plausible that ART may inhibit its activity, leading to differences in telomere lengths between ART-naïve and treated groups. Previous studies have produced inconsistent findings regarding such an association [30,31]. Comparison of levels in treated and ART-naïve groups in our study did not support this potential mechanism and furthermore, neither BoA was associated with ART duration.

Although we have demonstrated a substantial effect of HIV infection on biological aging, estimating the magnitude of the effect is challenging. One important reason is because disease states can cause stress induced premature senescence [26], leading to acute growth arrest (in contrast to gradual replicative senescence). Thus, the ‘rate of biological aging’ may not be accurately predicted in HIV infection. In other words, HIV-infected individuals effectively display a biological age observed in older uninfected individuals due to disease-induced stress. Therefore, quantification in terms of an effective biological age difference between infected and uninfected individuals is problematic as the component of stress induced premature senescence in infected individuals will be missing in uninfected controls, while in turn replicative senescence may be accelerated due to disease stress. Estimation of telomere length as a function of given chronological age has been quantified [32,33], however telomere length as an isolated measure at a given age may be imprecise, reflecting psychosocial, genetic and epigenetic confounders. The predictive capability of CDKN2A in determining effective biological age is relatively novel [9], and as CDKN2A expression is related to cellular growth arrest it may not be subject to similar attrition phenomena [16,23,25]. Future work should involve development of methods to establish quantification of differences in effective biological age in disease states where stasis is involved. Any measure of the difference in biological age between HIV-infected and HIV-seronegative individuals will also have to be viewed in a functional context between these two groups, where differences in biochemical parameters, such as interleukin-6, CD14 and D-dimer are also addressed [34].

A key strength of our study is the inclusion of an age- and gender-matched control group with a similar socio-demographic profile as the HIV-infected participants. The hypothesis of accelerated aging in HIV has received criticism due to limitations in characterization of participants, particularly the possibility of differential exposure to potential risk factors between HIV-infected and uninfected populations [35]. Since the HIV epidemic in South Africa is generalized and recruitment took place from the same community, the likelihood of differential exposure was minimized. The study design means that ‘survivor effects’ cannot be excluded. Individuals with poor biological aging may die earlier, thus participants comprise survivors with different biological characteristics to non-survivors. However, recruitment of individuals from similar socio-economic backgrounds should have limited potential confounding and survivor effects. The differences in smoking and alcohol consumption between the two groups could be due to HIV-infected participants modifying their smoking and alcohol consumption behaviours in response to lifestyle counselling. However, it is also plausible that the reported differences are due to misclassification, with HIV-infected participants wanting to demonstrate ‘healthy behaviour’, possibly leading to confounding. We used location of work as a proxy measure of ultra-violet exposure which may also have been confounded by socio-economic status. The gender composition of participants was three-quarters female, consistent with the characteristics of the clinic cohort from where our participants were enumerated [36], and reflective of the African AIDS epidemic. Lastly, as all our study participants are of African ancestry, our results are only generalizable to the African population.

The AIDS epidemic in sub-Saharan Africa is entering a new phase where HIV-infected individuals are living longer and may be aging faster [12,13]. Our data indicate increased biological aging in HIV-infected individuals compared to HIV-seronegative individuals. The increased number of older HIV-infected individuals in this region, compounded by accelerated biological aging may have wide-ranging implications for HIV management. Delivery of healthcare systems integrating HIV treatment and age-related morbidity may be necessary to manage this phase of the epidemic.

In light of these findings, important research questions arise: prospective assessment of biological age in HIV-infected and HIV-seronegative individuals is

needed to ascertain whether the accelerated aging trajectory develops as soon as HIV infection is acquired. Further questions include whether biological age is dependent upon the duration of untreated disease or nadir CD4 count, and if the biological age of the two groups continues to diverge during long-term ART, or rather is modified by ART. Finally, assessment of the relative contributions of HIV and ART towards biological aging may provide mechanistic insight.

In summary, greater biological age, as determined by shorter telomere length and higher CDKN2A expression, is associated with HIV infection in South Africa. The ‘aging of the HIV epidemic’ poses many challenges and these may be amplified by accelerated biological aging, potentially resulting in important health and social implications for the millions of patients receiving ART in Africa.

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Author contributions:

SP: conceived and designed experiments; undertook clinical data collection; statistical analysis; wrote first draft; critical revision and intellectual input to further drafts

SDL: designed experiments; critical revision and intellectual input to further drafts

CG: conceived and designed experiments; critical revision and intellectual input to further drafts

DM; LM: laboratory data analysis; critical revision and intellectual input to further drafts

HW: statistical analysis; critical revision and intellectual input to further drafts

JP; TC; KP: laboratory data analysis

LGB; RW: critical revision and intellectual input to further drafts

PGS: conceived and designed experiments; critical revision and intellectual input to further drafts

Conflicts of interest

All of the authors confirm there are no conflicts of interest to declare.

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Figure legends

Figure 1a: Measurement of telomere length in peripheral blood leukocytes in HIV-seronegative and HIV-infected individuals. Telomere length measured as mean relative T/S ratio: $RelT/S \pm SE$: 0.91 ± 0.007 vs. 1.07 ± 0.008 , $p < 0.0001$

Figure 1b: Measurement of CDKN2A expression in HIV-seronegative and HIV-infected individuals. Relative expression 0.45 ± 0.02 vs. 0.36 ± 0.03 , $p = 0.003$.

FIGURES AND TABLES

Figure 1a:

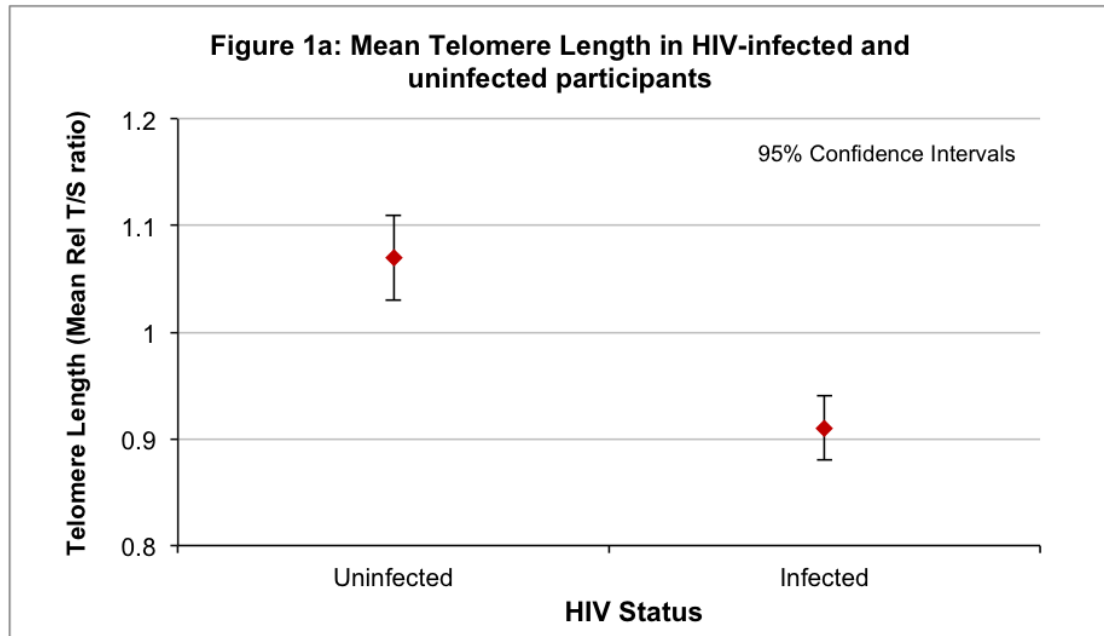


Figure 1b:

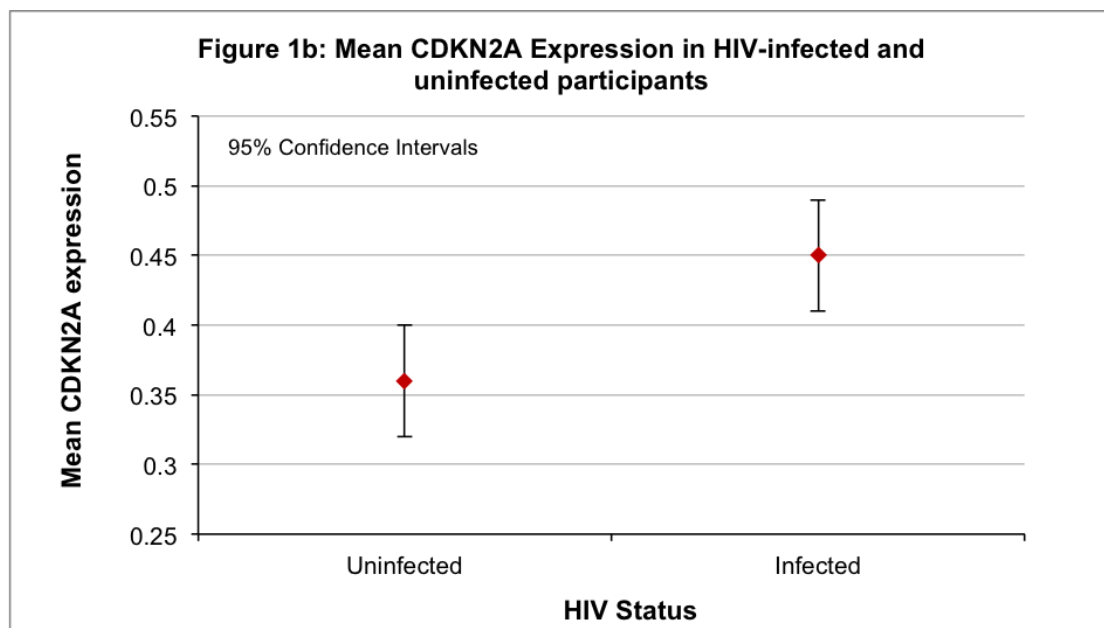


Table 1: Characteristics of study population

Variable	HIV-infected participants (236) % (n)	HIV-seronegative participants (250) % (n)	P-value
Age (years)	39 (35-46) ^a	40 (35-49) ^a	0.17
Age (years) by group			
30-39	50.4 (119)	46.4 (116)	
40-49	32.6 (77)	32.0 (80)	0.41
>50	17.0 (40)	21.6 (54)	
Male gender	25.4 (60)	24.0 (60)	0.72
Education			
< High school	11.9 (28)	17.6 (44)	0.08
High school or tertiary education	88.1 (208)	82.4 (206)	
Income			
<USD 125/month	56.8 (134)	67.2 (168)	0.02
>USD125/month	43.2 (102)	32.8 (82)	
Housing			
Informal	47.9 (113)	40.4 (101)	0.10
Formal	52.1 (123)	59.6 (149)	
Water supply			
Shared	75.0 (177)	74.0 (185)	0.80
Own	25.0 (59)	26.0 (65)	
Alcohol (amount per week)			
Nil	69.5 (164)	56.4 (141)	
Up to 1L/week	20.3 (48)	21.6 (54)	0.001
>1L/week	10.2 (24)	22.0 (55)	
Duration of smoking (years)			
Nil	84.8 (200)	72.0 (180)	
<10 years	5.1 (12)	12.8 (32)	0.002
>10 years	10.2 (24)	15.2 (38)	
Illicit drugs			
Never taken	97.4 (222)	98.0 (240)	
Ever taken	2.6 (6)	2.0 (5)	0.67

BMI (kg/m ²)		27.8±0.4	31.5±0.6	<0.0001
Co-morbidity (including hypertension)				
	None	64.4 (152)	55.2 (138)	
	One or more	35.6 (84)	44.8 (112)	0.04
TB status				
	No history	32.6 (77)	87.6 (219)	
	Current	4.2 (10)	0.4 (1)	
	Previous	63.1 (149)	12.0 (30)	<0.0001

HIV characteristics % (n) or median (IQR)

WHO stage		
	1/2	27.1 (67)
	3/4	72.3 (181)
ART naïve		12.9 (32)
CD4 count in ART naïve group (n=32)		182 (84-202)
(cells/μL)		
Log ₁₀ VL in ART naïve group (n=21)		4.88 (4.21-5.18)
Current CD4 count in ART group		468 (325-607)
(cells/μL)		
Nadir CD4 count in ART group		128 (76-171)
(cells/μL)		
% with undetectable VL* in ART group		84.3 (172)
Peak Log ₁₀ VL in ART group		4.56 (3.84-4.98)
Duration of ART, months		58 (34-75)

a – values expressed as median (interquartile range); *Undetectable VL refers to VL<50 copies/mL

Table 2: Association of HIV states with telomere length and CDKN2A expression

Clinical group	N	Telomere length Mean Rel T/S (95% CI)	P-value	N	Mean CDKN2A Mean expression (95% CI)	P-value
HIV-seronegative	250	1.07 (1.04-1.11)	<0.0001 ^a	217	0.35 (0.32-0.39)	0.006 ^a
HIV-infected; On ART	204	0.91 (0.87-0.94)		199	0.45 (0.40-0.50)	
HIV-infected; ART-naïve	32	0.89 (0.81-0.98)	0.71 ^b	28	0.46 (0.34-0.62)	0.80 ^b

a- indicates p-value for HIV-seronegative individuals vs. HIV-infected participants overall

b- indicates p-value between HIV group (i.e. on ART and ART-naïve)

Table 3: Association of biomarkers with HIV-related co-variates. Linear regression adjusted for age in participants on ART with suppressed VL

(i.e. <50 copies/mL) (*indicates P-value test for trend)

Variable	N =172	Telomere length Mean Rel T/S (95% CI)	P- value	N =168	Mean CDKN2A (95% CI)	P- value
WHO stage						
1/2	33	0.89 (0.81-0.97)		32	0.44 (0.35-0.56)	
3/4	139	0.92 (0.88-0.96)	0.48	136	0.46 (0.41-0.51)	0.81
Duration of ART, months						
0-36	40	0.86 (0.80-0.93)		39	0.51 (0.41-0.63)	
36-72	80	0.91 (0.87-0.97)	0.10*	79	0.45 (0.39-0.52)	0.17*
>72	52	0.95 (0.88-1.01)		50	0.42 (0.35-0.51)	
Current CD4 count (cells/μL)						
<200	7	0.85 (0.70-1.02)		7	0.63 (0.38-1.03)	
201-350	37	0.83 (0.77-0.91)	0.02*	37	0.52 (0.42-0.64)	0.05*
>351	128	0.93 (0.90-0.98)		124	0.43 (0.38-0.48)	
Nadir CD4 count (cells/μL)						
<200	153	0.91 (0.87-0.94)		151	0.46 (0.41-0.51)	0.80
>201	19	0.93 (0.83-1.05)	0.58	17	0.44 (0.32-0.61)	
Peak viral load						
<10,000 copies	52	0.92 (0.86-0.99)		51	0.50 (0.42-0.61)	
>10,000 copies	120	0.91 (0.87-0.95)	0.68	117	0.44 (0.38-0.48)	0.27
TB status						
No history	48	0.91 (0.84-0.97)		47	0.47 (0.39-0.57)	
Current /Previous history	124	0.91 (0.87-0.95)	0.92	121	0.45 (0.40-0.51)	0.69

Table 4a: Mean telomere length (RelT/S) in HIV-infected participants and HIV-seronegative individuals, adjusted for age (*indicates P-value test for trend)

Variable	HIV-infected participants			HIV-seronegative participants		P-value
	N	Mean Rel T/S (95% CI)	P	N	Mean Rel T/S (95% CI)	
Age, years						
30-39	119	0.92 (0.88-0.96)		116	1.10 (1.04-1.15)	
40-49	77	0.93 (0.88-0.98)	0.11	80	1.09 (1.02-1.15)	0.09*
>50	40	0.84 (0.77-0.91)		54	1.01 (0.94-1.08)	
Gender						
Male	60	0.92 (0.86-0.99)		60	1.04 (0.97-1.12)	
Female	176	0.90 (0.87-0.94)	0.56	190	1.08 (1.04-1.12)	0.30
Income/month (US\$)						
<USD 125/month	134	0.91 (0.87-0.95)		168	1.07 (1.02-1.12)	
>USD125/month	102	0.90 (0.85-0.95)	0.86	82	1.07 (1.00-1.14)	0.94
UV exposure/ Occupation						
Outdoor worker	155	0.90 (0.86-0.94)		178	1.08 (1.04-1.13)	
Indoor worker	81	0.92 (0.87-0.97)	0.69	72	1.04 (0.98-1.12)	0.30
Education						
< High school	28	0.91 (0.82-1.01)		44	1.04 (0.96-1.11)	
High school /college	208	0.91 (0.87-0.94)	0.91	206	1.08 (1.04-1.12)	0.49
Housing						
Formal	123	0.91 (0.87-0.96)		149	1.06 (1.02-1.11)	
Informal	113	0.90 (0.86-0.95)	0.69	101	1.09 (1.03-1.15)	0.77
Duration of smoking						
Nil	200	0.91 (0.88-0.95)		180	1.08 (1.03-1.12)	
<10 years	12	0.93 (0.80-1.08)	0.36	32	1.07 (0.97-1.17)	0.92
≥10 years	24	0.85 (0.77-0.95)		38	1.07 (0.98-1.16)	
Alcohol (per week)						
Nil	164	0.92 (0.86-0.96)		141	1.08 (1.03-1.13)	
<1L	48	0.89 (0.82-0.95)	0.18*	54	1.13 (1.05-1.22)	0.04
>1L	24	0.86 (0.77-0.95)		55	1.00 (0.93-1.08)	

Body mass index							
(kg/m ²)							
	<25	87	0.88 (0.83-0.93)		60	1.04 (0.97-1.11)	
	25-29.9	71	0.96 (0.90-0.98)	0.08	55	1.12 (1.04-1.20)	0.35
	>30	78	0.90 (0.85-0.95)		135	1.07 (0.98-1.12)	
Co-morbid condition							
	No	152	0.91 (0.87-0.95)		138	1.08 (1.04-1.14)	
	Yes	84	0.90 (0.85-0.95)	0.88	112	1.06 (1.00-1.11)	0.65
TB status							
	No history	77	0.90 (0.85-0.96)	0.88	219	1.07 (1.03-1.11)	0.71
	Current/past	159	0.91 (0.87-0.95)		31	1.09 (0.99-1.20)	

Table 4b: Relative CDKN2A expression in HIV-infected participants and HIV-seronegative individuals, adjusted for age. (*indicates P-value test for trend)

Variable	HIV-infected participants			HIV-seronegative participants		
	N	Mean CDKN2A	P-value	N	Mean CDKN2A	P-value
Age, years						
30-39	113	0.41 (0.36-0.47)		98	0.29 (0.25-0.35)	
40-49	74	0.45 (0.38-0.53)	0.04*	70	0.44 (0.37-0.54)	0.006
>50	40	0.54 (0.43-0.68)		49	0.38 (0.30-0.48)	
Gender						
Male	59	0.42 (0.35-0.50)		53	0.37 (0.30-0.47)	
Female	168	0.45 (0.41-0.51)	0.46	163	0.35 (0.31-0.40)	0.71
Income/month (US\$)						
<USD 125/month	129	0.43 (0.38-0.48)		145	0.39 (0.34-0.44)	
>USD125/month	98	0.47 (0.41-0.54)	0.44	72	0.30 (0.23-0.37)	0.03
UV exposure/Occupation						
Outdoor worker	147	0.45 (0.40-0.50)		155	0.36 (0.32-0.41)	
Indoor worker	80	0.45 (0.38-0.52)	0.81	62	0.34 (0.28-0.42)	0.86
Education						
< High school	27	0.48 (0.36-0.64)		39	0.34 (0.26-0.44)	
High school /college	200	0.44 (0.40-0.49)	0.60	178	0.36 (0.32-0.41)	0.66
Housing						
Formal	121	0.44 (0.39-0.50)		126	0.34 (0.30-0.40)	
Informal	106	0.45 (0.40-0.52)	0.63	91	0.37 (0.31-0.44)	0.47
Duration of smoking						
Nil	192	0.45 (0.40-0.49)		157	0.36 (0.31-0.40)	
<10 years	12	0.65 (0.43-0.93)		27	0.43 (0.31-0.58)	
>10 years	23	0.36 (0.28-0.49)	0.08	33	0.30 (0.22-0.43)	0.23
Alcohol (amount per week)						
Nil	155	0.45 (0.40-0.50)		126	0.35 (0.30-0.40)	
<1L	48	0.44 (0.36-0.54)		42	0.36 (0.28-0.47)	
>1L	24	0.41 (0.31-0.55)	0.72	49	0.37 (0.29-0.47)	0.87

Body mass index (kg/m ²)							
<25	82	0.41 (0.36-0.49)		55	0.33 (0.26-0.41)		
25-29.9	69	0.49 (0.41-0.57)		46	0.38 (0.30-0.48)		
>30	76	0.45 (0.38-0.52)	0.44	116	0.36 (0.31-0.42)		0.59
Co-morbid condition							
No	144	0.44 (0.38-0.48)		119	0.39 (0.34-0.46)		
Yes	83	0.46 (0.41-0.55)	0.73	98	0.32 (0.26-0.38)		0.08
TB status							
No history	73	0.45 (0.38-0.53)	0.93	191	0.36 (0.32-0.40)		0.80
Current/past	154	0.44 (0.40-0.50)		26	0.34 (0.25-0.48)		

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Chapter 6

Frailty in HIV-infected individuals in South Africa



Participants undergoing evaluation of frailty status; participant interview (top), measuring walking time (lower left), assessing grip strength (lower right)

Research paper assessing prevalence and predictors of frailty in HIV-infected individuals

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? ***Journal of Acquired Immunodeficiency Syndromes (JAIDS)***
 - 1.2. When was the work published? ***E-pub: Sept 26th. Paper version Jan 2013***
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion ***N/A***
 - 1.3. Was the work subject to academic peer review? ***Yes***
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See Appendix 7
2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? ***N/A***
 - 2.2. List the paper's authors in the intended authorship order
 - 2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I conceived the idea for the experiments. I designed the experiments and conducted the data collection with assistance from the research study nurse. I performed the statistical analyses with advice from Helen Weiss. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



Frailty in HIV-Infected Adults in South Africa

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Objectives: Some evidence suggests that HIV infection is associated with premature frailty—a syndrome typically viewed as being related to ageing. We determined the prevalence and predictors of frailty in a population of HIV-infected individuals in South Africa.

Design: Case-control study of 504 adults more than the age of 30 years, composed of 248 HIV-infected adults and 256 age- and gender-matched, frequency-matched HIV-seronegative individuals.

Methods: Frailty was defined by standardized assessment comprised of ≥ 3 of weight loss, low physical activity, exhaustion, weak grip strength, and slow walking time. Independent predictors of frailty were evaluated using multivariable logistic regression.

Results: The mean ages of the HIV-infected and HIV-seronegative groups were 41.1 ± 7.9 years and 42.6 ± 9.6 years, respectively. Of the HIV-infected adults, 87.1% were receiving antiretroviral treatment (median duration, 58 months), their median CD4 count was 468 cells/ μ L (interquartile range = 325–607 cells/ μ L) and 84.3% had undetectable plasma viral load. HIV-infected adults were more likely to be frail than HIV-seronegative individuals (19.4% vs. 13.3%; $P = 0.07$), and this association persisted after adjustment for confounding variables [adjusted OR = 2.14; 95% confidence interval

(95% CI): 1.16–3.92, $P = 0.01$]. Among HIV-infected individuals, older age was a strong predictor of frailty, especially among women (women: OR = 2.55 per 10-year age increase; men: OR = 1.29 per 10-year age increase, P -interaction = 0.01). Lower current CD4 count (< 500 cells/ μ L) was also independently associated with frailty (OR = 2.84; 95% CI: 1.02–7.92, $P = 0.04$).

Conclusions: HIV infection is associated with premature development of frailty, especially in women. Since higher CD4 counts were associated with lower risk of frailty, earlier initiation of antiretroviral treatment may be protective.

Key Words: HIV, AIDS, frailty, premature ageing, South Africa

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INTRODUCTION

Major reductions in HIV-associated mortality have occurred as a result of the global scale-up of highly active antiretroviral therapy (ART). This is largely due to prevention of AIDS-related events but is also due to a decrease in non-AIDS-related events and deaths.^{1–3} Despite these benefits, evidence is emerging that patients receiving ART are at an increased risk of age-related non-AIDS morbidity and mortality compared with HIV-seronegative individuals.^{4–6} Several of these conditions are classically associated with the normal ageing process but seem to occur at an earlier age in HIV-infected persons compared with age-matched HIV-seronegative individuals. It is possible that not only are HIV cohorts ageing chronologically, but they may also be undergoing accelerated physiologic and immunologic senescence.

Frailty is a clinical syndrome initially described in geriatric populations. It reflects a concept of decreased physiologic and functional reserve and a subsequent decrease in adaptation to external or intrinsic stressors. Frailty is characterized by multiple pathologies, low physical activity, and slow motor performance,^{7,8} and leads to cognitive and physical decline manifest as an increased risk of mortality, falls, and hospitalization. HIV infection has been associated with premature development of frailty, and it has been speculated that this may emerge as an important clinical syndrome in HIV-infected individuals. The prevalence of premature presentation of frailty in HIV-infected populations is reported to range between 5% and 20%, depending on the study population.^{9–12} However, limitations of these reports include studying single sex cohorts and differences in frailty criteria. There is also substantial potential for confounding because of differential exposure to potential risk factors between the study

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population and the reference uninfected population (if used), thereby altering their risk of age-related outcomes.

The epidemiology of HIV and AIDS in sub-Saharan Africa is changing; extensive ART scale-up has led to reduced mortality rates and a rapidly expanding cohort of HIV-infected African patients who are living much longer than they were previously.^{13,14} However, it is unclear whether these individuals will also be subject to premature ageing in the longer term. Estimates from previous studies from Europe and North America where the epidemiology of HIV differs substantially from that in sub-Saharan Africa, may not be generalizable to African HIV cohorts. In this study, we determined the prevalence and predictors of frailty in an HIV-infected population in sub-Saharan Africa.

METHODS

Study Participants

Between March and December 2011, unselected HIV-infected individuals older than 30 years were enrolled from a community-based HIV treatment center in Nyanga district of Cape Town, which has been previously described.^{15,16} All participants had a confirmed serological diagnosis of HIV and were either about to commence ART (ART-naïve) or were already on first-line ART. Participants who had active opportunistic infections (OIs) were not recruited; however, participants who had active tuberculosis (TB) (ie, non-symptomatic but still receiving treatment for TB) were enrolled.

A control group of HIV-uninfected participants was recruited using frequency matching by gender and 5-year age categories. HIV-seronegative individuals were enrolled from participants confirmed to be HIV-negative attending an HIV prevention trials site (Emavundleni Centre), located within the same district as the HIV treatment center. These 2 centers were chosen as individuals attending them were drawn from the same community and were therefore likely to have similar sociodemographic characteristics.

The study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee and the University of Cape Town Faculty of Health Sciences Research Ethics Committee and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Assessment of Frailty and Data Collection

Physical frailty was defined by the presence of 3 or more of the 5 criteria: (1) unintentional weight loss (self-report of weight loss was verified in 100 (20%) participants by weighing of the participant at the time of data collection followed by referral to clinic records. Self-report and clinical records were congruent in 80 participants), (2) self-reported low physical activity, (3) self-reported exhaustion, (4) weak grip strength, and (5) slow walking time (see **Appendix, Supplemental Digital Content**, <http://links.lww.com/QAI/A358>). All of these 5 components described in the original phenotype by Fried et al¹⁷ were used to determine the presence of frailty. However, we used the proxy described by Onen et al¹⁰ for the physical activity measure

(for description, see **Appendix, Supplemental Digital Content**, <http://links.lww.com/QAI/A358>). Grip strength of the dominant hand was measured 3 times using a grip dynamometer (Jamar Plus+ Digital Hand Dynamometer, Jamar, Sammons Preston, Rolyan, Bolingbrook, IL). The average of 3 weight measurements was recorded in kilograms (kg) to one decimal point. Walking time was assessed using the method of Cesari et al.¹⁸ The average of 2 trials (in meters per second) was used for analysis. Participants were excluded from the determination of grip strength if they had pain or arthritis of the dominant hand and excluded from the walking test if they had paralysis of an extremity or side of the body or needed to use a walking aid.

Sociodemographic information and medical history were obtained via a questionnaire administered in the participant's first language (Xhosa or English). Sociodemographic and behavioral variables of interest included education, alcohol consumption, smoking history, and income (salaried income and/or social welfare grant). Clinical information was obtained from medical case notes where required. Comorbidity was defined as the concurrent presence of one or more chronic diseases or conditions, including cardiovascular disease, chronic renal failure, airways disease, and malignancy (both AIDS and non-AIDS defining). Cardiovascular diseases included myocardial infarction and cerebrovascular disease. Blood pressure (BP) was measured using a digital sphygmomanometer. Hypertension was defined as a systolic BP of 140 mm Hg or higher, diastolic BP of 90 mm Hg or higher, or the combination of self-reported high BP diagnosis and the use of antihypertensive medications. Height was measured in meters, weight in kilograms, and body mass index (BMI) was calculated as weight per height.² HIV-related conditions were classified according to the WHO staging system and were based on historical assessment done at the time of enrolment into the ART service. ART was defined as the use of 3 or more antiretroviral drugs, and treatment duration was recorded in months. Nadir and current CD4 count and HIV RNA plasma viral load (VL) were available from medical records, current values being measured within an 8-week period before frailty assessment. VL suppression was defined as HIV RNA <50 copies/mL.

Statistical Analysis

Analyses were conducted on participants with criteria available for determination of frailty phenotype (3 or more of the criteria present). Participants were categorized as "frail" and "non-frail," using the criteria given above. Comparisons between categorical groups were performed using χ^2 tests. Continuous variables were compared using Student *t* test if normally distributed and the Mann-Whitney *U* test if non-normally distributed. Variables were log transformed where appropriate. All *P* values were 2-tailed and considered significant if *P* < 0.05. Univariable logistic regression was performed to estimate odds ratio (OR) and 95% confidence intervals (CIs) of factors associated with frailty. Multivariable logistic regression was then used to evaluate independent factors associated with frailty overall and within the case and control groups, respectively. HIV-infected individuals were also analyzed based on their ART status (naïve or on

TABLE 1. Characteristics of Study Population

Variable	HIV-Infected (248), % (n)	HIV-Seronegative (256), % (n)	P
Age (yrs), mean ± SD	41.1 ± 7.9	42.6 ± 9.6	0.07
Age (yrs) by group			
30–39	49.6 (123)	49.1 (118)	
40–49	33.1 (82)	32.8 (84)	0.09
50–59	15.3 (38)	14.5 (37)	
>60	2.0 (5)	6.7 (17)	
Male gender	26.6 (66)	25.0 (64)	0.68
Education			
<High school	12.1 (30)	18.4 (47)	0.05
Income			
<USD 125/mo	58.4 (145)	68.0 (174)	0.03
Marital status			
Single	68.6 (170)	63.7 (163)	
Married	25.4 (63)	29.3 (75)	0.68
Divorced	1.6 (4)	2.3 (6)	
Widowed	4.4 (11)	4.7 (12)	
Housing			
Informal	47.2 (117)	40.6 (104)	0.14
Water supply			
Shared	74.2 (184)	73.8 (189)	0.93
Alcohol (amount per week)			
Nil	70.5 (172)	54.4 (136)	
Up to 1 L/wk	18.4 (45)	22.8 (57)	<0.0001
>1 L/wk	11.1 (27)	22.8 (57)	
Duration of smoking (yrs)			
Nil	84.7 (210)	72.3 (185)	
5 yrs or less	2.4 (6)	7.4 (19)	0.008
6–15	3.2 (8)	7.0 (18)	
16–20	4.4 (11)	6.3 (16)	
>20	5.2 (13)	7.0 (18)	
Illicit drugs			
Ever taken	2.5 (6)	2.4 (6)	0.94
BMI (kg/m ²)	27.7 ± 6.5	31.3 ± 8.8	<0.0001
Comorbidity			
None	64.5 (160)	56.3 (144)	
One or more	35.5 (88)	43.7 (112)	0.06
TB status			
No history	31.9 (79)	87.9 (225)	<0.0001
Current	4.0 (10)	0.4 (1)	
Previous	64.1 (159)	11.7 (30)	
Number of pregnancies (n = 374)			
2 or less	62.1 (113)	47.9 (92)	
≥3	37.9 (69)	52.1 (100)	0.006
HIV characteristics (n = 248)	Median (IQR) or % (n)		
WHO stage			
1/2	27.1 (67)		
3/4	72.3 (181)		
ART naive	12.9 (32)		

TABLE 1. (Continued) Characteristics of Study Population

HIV characteristics (n = 248)	Median (IQR) or % (n)
CD4 count in ART naive group (n = 32)	182 (84–202)
Log ₁₀ VL in ART naive group (n = 21)	4.88 (4.21–5.18)
Current CD4 count in ART group	468 (325–607)
Nadir CD4 count in ART group	128 (76–171)
Percentage with undetectable VL in ART group	84.3 (182)
Peak Log ₁₀ VL in ART group	4.56 (3.84–4.98)
Duration of ART, months	58 (34–75)
ART regimen	
Containing AZT/3TC	59.7 (129)
Other	40.3 (87)

treatment). All analyses were performed with Stata 11 (Stata Corp, College Station, TX).

RESULTS

Participant Characteristics

Of the 504 participants, 248 had HIV-infection and 256 were HIV-seronegative individuals. The 2 groups of participants were similar in terms of age and gender (Table 1), but HIV-infected individuals tended to have higher levels of education (87.9% vs. 81.6%, *P* = 0.05) and reported lower levels of alcohol use and smoking consumption than HIV-seronegative individuals (*P* < 0.05). HIV-infected individuals reported a greater income than HIV-seronegative individuals (41.6% vs. 32.0% received more than ZAR1000 a month (approximately USD125), which was related to a greater proportion of the HIV-infected group receiving social welfare grants compared with HIV-seronegative individuals (data not shown). Although TB in the form of current or past disease was more common in the HIV group, other comorbidity was slightly higher in the control group. BMI was lower in HIV-infected individuals compared with HIV-seronegative individuals (27.7 ± 6.5 vs. 31.3 ± 8.8 kg/m², *P* < 0.0001). Among females, HIV-infected individuals reported fewer pregnancies than HIV-seronegative individuals (*P* = 0.006).

Clinical characteristics of the HIV-infected individuals are also reported in Table 1. Overall, 72.9% had a history of WHO stage 3 or stage 4 defining illness. The current CD4 count among participants receiving ART was 468 cells/μL [interquartile range (IQR) = 325–607 cells/μL] and 84.3% had undetectable VL. Median treatment duration on ART was 58 months (IQR, 34–75 months). About 12.9% of the HIV-infected group were ART naive and had correspondingly lower CD4 counts and higher VL (Table 1).

Prevalence of Frailty and Association With HIV

Assessment of frailty was possible in all participants (n = 504, with 3 participants excluded from the walking test but still contributing frailty data). Frailty outcomes within

TABLE 2. Frailty-Related Outcomes Within Study Population

Frailty Outcome	HIV-Infected (248), % (n)	HIV-Seronegative (256), % (n)	P
Frailty levels			
Robust (0)	31.8 (78)	35.9 (92)	
Prefrail (1–2)	49.2 (122)	50.8 (130)	0.16
Frail (3+)	19.4 (48)	13.3 (34)	
Overall frailty (3+ from 5)			
No	80.7 (200)	86.7 (222)	0.065
Yes	19.4 (48)	13.3 (34)	
Contribution to frailty criteria	Frailty + HIV+ (48)	Frailty+ HIV– (34)	
Exhaustion	95.8 (46)	94.1 (32)	0.72
Low physical activity	83.3 (40)	91.1 (31)	0.31
Weight loss	64.6 (31)	47.1 (16)	0.11
Weak grip strength	43.8 (21)	58.8 (20)	0.18
Slow walking time	58.3 (28)	47.1 (16)	0.31

the study population are reported in Table 2. The prevalence of frailty was greater among HIV-infected individuals than HIV-seronegative individuals (19.4%, 95% confidence interval [CI]: 14.4% to 24.3% vs. 13.3%, 95% CI: 9.1% to 17.5%; $P = 0.07$). Levels of “prefrailty” (ie, scoring 1 or 2 of the frailty criteria) were similar between the 2 groups. Of the frailty indicators, weight loss and slow walking time were more common in the HIV-infected group, however, these differences did not reach statistical significance.

Multivariable analysis adjusting for baseline characteristics and a priori confounders showed that HIV infection was independently associated with frailty (adjusted OR = 2.14; 95% CI: 1.16 to 3.92; Table 3). Age was also a strong predictor, and this association was stronger in females (P for interaction = 0.03). A higher level of education reduced the odds of frailty (OR = 0.44; 95% CI: 0.21 to 0.90). Low BMI was also strongly associated with frailty. TB was not included in the model because of its high correlation with HIV status. When TB was analyzed as a potential binary outcome of interest (no history vs. current/past) the association with frailty was not as strong as that for HIV (OR = 1.73; 95% CI: 0.94 to 3.08) and did not reach statistical significance.

Predictors of Frailty in the HIV-Infected Group

Among the 216 HIV-infected individuals on ART, the prevalence of frailty was 18.0% (95% CI: 13.2% to 23.8%). In univariable analyses, older age and lower socioeconomic status (including education level) and alcohol consumption were associated with higher odds of frailty.

In the multivariable model, there was evidence of an interaction between gender and age with a strong association between increasing age and frailty within females but not males (P for interaction = 0.001). Low BMI was also a strong predictor of frailty (P trend = 0.01). Among the HIV-related covariates, a current CD4 count less than 500 cells/ μ L was

TABLE 3. Multivariable Logistic Regression Model to Identify Predictors of Frailty in All Study Participants (n = 504)*

Variable	OR	P
HIV	2.14 (1.16–3.92)	0.01
Sex		
Male	1	
Female	1.29 (0.41–4.08)	0.03 for interaction
Male: (per 10 yr increase)	0.94 (0.41–2.15)	
Female (per10 yr increase)	2.55 (1.75–3.71)	
Alcohol (amount per week)		
Nil	1	
<500 mL	0.75 (0.22–2.60)	0.10
500 mL–1 L	0.57 (0.24–1.39)	
>1 L	0.24 (0.08–0.77)	
Education		
<High school	1	
High school/college	0.44 (0.21–0.90)	0.02
BMI (kg/m ²)		
<20	1	
20–24.9	0.22 (0.08–0.61)	
25–29.9	0.21 (0.08–0.60)	0.02
>30	0.20 (0.07–0.58)	
BP		
None	1	
Hypertensive	0.83 (0.45–1.53)	0.54
Comorbid condition		
No	1	
Yes	3.20 (0.76–13.50)	0.11
Duration of smoking (yrs)		
Nil	1	
5 yrs or less	0.78 (0.15–4.04)	
6–15	2.70 (0.63–11.52)	0.37
16–20	2.48 (0.79–7.77)	
>20	0.76 (0.16–3.59)	
Income/month (ZAR)		
<USD 125/mo	1	
>USD125/mo	1.05 (0.58–1.90)	0.87
Occupation		
Unemployed/grant	1	
Employed	0.65 (0.35–1.20)	0.17
Housing		
Formal	1	
Informal	0.70 (0.40–1.24)	0.23

*Adjusted for all variables displayed within table.
USD, US dollars.

associated with increased risk of frailty (OR = 2.84; 95% CI: 1.02 to 7.92). No associations were found with HIV RNA levels (current or nadir), duration of treatment, or ART regimen. Socioeconomic variables were not associated with frailty in this model. In a multivariable analysis restricted to females on ART, a similar association was seen with current CD4 count. Age continued to act as a strong predictor and low BMI. A history of 3 or more pregnancies was also associated with frailty in this group [OR = 3.42 (1.03–11.36), $P = 0.04$] (data not shown).

TABLE 4. HIV Predictors of Frailty in HAART HIV+ Group (n = 216)*

Variable	Unadjusted OR	P	Fully Adjusted OR	P
Sex				
Male	1		Males	1
Female	1.65 (0.68–4.00)	0.27	Female	0.66 (0.13–3.22)
Age, yrs			Male (per 10 yr increase)	0.37 (0.08–1.77)
30–39	1		Female (per 10 yr increase)	2.50 (1.35–4.58)
40–40	1.10 (0.45–2.68)			
50–59	5.90 (2.40–14.48)	0.0008		
>60	2.36 (0.23–24.40)			
HIV-related characteristics				
WHO clinical stage				
1/2	1		1	
3/4	1.10 (0.47–2.57)	0.83	1.19 (0.26–5.38)	0.83
Duration of ART, mo				
0–36	1		1	
36–72	0.42 (0.18–0.98)		0.49 (0.16–1.49)	
>72	0.80 (0.34–1.85)	0.11	1.03 (0.31–3.41)	0.30
Current CD4 count, cells/μL				
<500	1.77 (0.84–3.72)	0.12	2.84 (1.02–7.92)	
>500	1		1	0.04
Nadir CD4 count, cells/μL				
<200	0.62 (0.23–1.69)	0.37	0.42 (0.11–1.56)	
>201	1		1	0.19
Current Log ₁₀ VL				
<10,000 copies	1		1	
>10,000 copies	0.82 (0.17–3.83)	0.80	0.41 (0.05–3.63)	0.40
Peak Log ₁₀ VL				
<10,000 copies	1		1	
>10,000 copies	1.39 (0.62–3.12)	0.42	2.38 (0.82–6.92)	0.11
ART Regimen				
Containing AZT/3TC	1		1	
Other	1.03 (0.51–2.10)	0.91	0.60 (0.24–1.51)	0.28
TB status				
No history	1		1	
Current infection	1.48 (0.15–15.66)	0.93	0.65 (0.02–17.61)	0.96
Previous history	0.96 (0.44–2.09)		0.89 (0.22–3.54)	
Demographic characteristics				
BMI (kg/m ²)				
<20	1		1	
20–24.9	0.60 (0.18–2.03)		0.29 (0.06–1.36)	
25–29.9	0.53 (0.16–1.80)	0.48	0.18 (0.03–0.99)	0.01
>30	0.38 (0.11–1.31)		0.09 (0.01–0.55)	p-trend
Comorbid condition (non-HIV related)				
No	1		1	
Yes	1.01 (0.48–2/15)	0.97	0.67 (0.25–1.77)	0.42
Income per month (US dollars)				
<USD 125/mo	1		1	
>USD125/mo	1.26 (0.63–2.53)	0.51	1.34 (0.57–3.16)	0.48
Occupation				
Unemployed/welfare grant	1		1	
Employed	0.50 (0.23–1.06)	0.07	0.54 (0.21–1.36)	0.23
Education				
<High school	1		1	

(continued on next page)

TABLE 4. (Continued) HIV Predictors of Frailty in HAART HIV+ Group (n = 216)*

	Unadjusted OR	P	Fully Adjusted OR	P
High school/college	0.33 (0.13–0.82)	0.02	0.76 (0.21–2.82)	0.69
Housing				
Formal	1		1	
Informal	0.91 (0.45–1.82)	0.78	0.52 (0.23–1.28)	0.16
Alcohol (amount per week)				
Nil	1		1	
<500 mL	0.88 (0.18–4.32)		1.40 (0.15–12.18)	
500 mL–1 L	0.26 (0.06–1.15)	0.03	0.17 (0.03–1.10)	0.07
>1L	0.15 (0.02–1.17)		0.08 (0.01–0.88)	p-trend
Duration of smoking				
Nil	1		1	
<10 yrs	0.47 (0.06–3.86)		1.20 (0.09–15.92)	
>10 yrs	0.67 (0.19–2.40)	0.62	0.78 (0.13–4.67)	0.95

*Fully adjusted model adjusted for all variables displayed within table. USD, US dollars.

In the ART naive group (n = 32), 28.1% (95% CI: 13.7% to 46.7%; n = 9) were frail. The only factor associated with frailty in a multivariable analysis was WHO clinical stage 3 or 4. Age and gender and other HIV-related covariates were not associated.

To assess the possibility of current diagnoses of TB (the most common OI in this setting)¹⁵ causing false misclassification of frailty, we constructed a separate multivariable model excluding such patients (n = 10). In this model, HIV still remained strongly predictive of frailty (OR = 2.08; 95% CI: 1.12 to 3.86, P = 0.02). In a further model of HIV-infected participants that similarly excluded those with a current diagnosis of TB, CD4 count remained a strong predictor of frailty (OR = 2.89; 95% CI: 1.02 to 7.92, P = 0.04) (Table 4).

Predictors of Frailty in the Control Group

Among HIV-seronegative individuals, frailty was independently associated with older age, female gender, and smoking (Table 5). There was also some evidence that TB was associated with frailty. In view of the strong association of female gender with frailty, we performed subanalyses restricted to females. In adjusted analyses, increasing age was still a strong predictor of frailty.

DISCUSSION

This study provides clear evidence that HIV infection is strongly associated with a 2-fold risk of premature frailty in this African population. Premature frailty was most prevalent in women who comprise the majority of the HIV-infected population in sub-Saharan Africa. These findings have potentially important implications for long-term morbidity among the millions of patients receiving ART long-term in Africa and may have an important bearing on the optimum timing of ART initiation.

Our HIV-related frailty prevalence of 19.4% is higher than estimates from other regions. A prevalence of 9% was reported from a clinic population in the United States (mean

age of 42 years).¹⁰ The Women's Interagency HIV Study (a prospective cohort in 5 US cities) found a prevalence of 12% in HIV-infected women with clinical AIDS (median age of 41 years).¹¹ In the Multicenter AIDS Cohort Study (a longitudinal study of men who have sex with men), a frailty prevalence of 5%–14% depending on age and duration of HIV infection was reported from 1994–2005 data^{9,12} and 8% in 2009–2010 among men aged 40–49 years.¹⁹ In the SUN study (a US observational cohort of HIV-infected adults, median age of 47 years), 5% of participants were frail.²⁰ The variation in estimates is likely to be attributable to differences in study design and clinical demographics of patients recruited (eg, ART status and degree of immunodeficiency).

In agreement with previous studies of participants on ART, current CD4 count was a strong independent predictor of frailty.^{9,11,12,19} However, we did not find an association with duration of ART or nadir CD4 cell count. These findings are entirely consistent with previous analyses of factors associated with the risk of incident TB and of mortality in this cohort.^{21,22} Thus, although these 3 variables are interrelated,²³ it seems that current CD4 count best captures current “well-being” in this ART cohort. Thus, the best way to prevent frailty may be to maintain high CD4 counts through early initiation of ART. Potential reductions in non-AIDS-related morbidity and mortality from earlier initiation of ART are currently under investigation in the Strategic Timing of AntiRetroviral Treatment study²⁴ and prevention of frailty may be another important benefit of such a strategy.

It is possible that frailty status could be misclassified in some HIV-infected participants because of recent or current OIs. However, we think this unlikely for several reasons. Participants with acute symptomatic OIs were not eligible for enrolment in the study and a large majority of patients were clinically stable on long-term ART (median 58 months). They had a median CD4 count of 468 cells/μL and so were at low risk of current comorbidity, and previous WHO stage 3 and 4 defining illnesses were remote. A small number of participants had current diagnoses of TB for which continuation

TABLE 5. Predictors of Frailty in Uninfected Control Group (n = 256)*

Variable	Unadjusted OR	P	Adjusted OR	P
Age, yrs				
30–39	1		1	
40–49	6.39 (1.74–23.41)	<0.0001	8.59 (2.03–36.32)	0.0006
50–59	14.20 (3.65–55.12)		8.71 (1.75–43.31)	
>60	43.13 (9.71–191.40)		24.10 (3.19–182.20)	
Sex				
Male	1		1	
Female	3.95 (1.15–13.27)	0.03	11.03 (1.68–72.37)	0.01
Income per month (US dollars)				
<USD 125/mo	1		1	
>USD 125/mo	1.82 (0.88–3.81)	0.10	0.92 (0.28–1.93)	0.88
Occupation				
Unemployed/welfare grant	1		1	
Employed	0.45 (0.20–1.04)	0.06	0.64 (0.21–1.93)	0.44
Housing				
Formal	1		1	
Informal	0.48 (0.21–1.08)	0.09	0.45 (0.16–1.25)	0.13
Duration of smoking				
Nil	1		1	
<10 yrs	0.20 (0.02–1.53)	0.05	0.67 (0.13–3.52)	0.03
>10 yrs	1.71 (0.79–4.14)		6.01 (1.51–23.85)	
Alcohol (amount per week)				
Nil	1		1	
<500 mL	0.98 (0.20–4.78)	0.12	1.04 (0.16–6.86)	0.92
500 mL–1 L	0.61 (0.22–1.72)		1.31 (0.34–5.06)	
>1 L	0.27 (0.08–0.95)		0.67 (0.13–3.53)	
Education				
<High school	1		1	
High school/college	0.34 (0.16–0.77)	0.008	0.47 (0.17–1.32)	0.15
BMI (kg/m ²)				
<20	1		1	
20–24.9	0.35 (0.15–0.82)	0.10	0.07 (0.01–1.11)	0.64
25–29.9	0.42 (0.19–0.94)		0.28 (0.03–2.39)	
>30	0.43 (0.21–0.90)		0.40 (0.05–3.11)	
TB status				
No history	1		1	
Current/previous infection	1.68 (0.63–4.48)	0.29	3.65 (0.96–13.81)	0.06
Hypertension				
Normotensive	1		1	
Hypertensive	2.79 (1.31–5.93)	0.008	1.09 (0.38–3.09)	0.87
Comorbid condition				
No	1		1	
Yes	4.8 (1.28–18.00)	0.02	3.04 (0.47–19.86)	0.24

*Fully adjusted model adjusted for all variables displayed within table.
USD, US dollars.

phase treatment was being received. Estimates of HIV as a predictor of frailty did not greatly change when these participants were excluded from analyses, suggesting that misclassification was minimal. Thus, we believe that the observed association of frailty with the HIV-infected group is likely to be related to HIV infection itself rather than as a consequence of symptoms related to current OIs.

Female gender was an important predictor of frailty in both study populations. In the general population, frailty is

more common among females.^{25,26} Men may be protected by greater muscle mass and higher testosterone levels^{27,29} reflecting the greater biological capital they achieve before age-related decline. Two US-based studies did not find an association of female gender with frailty,^{10,20} possibly due to the gender composition (predominantly male) of the study populations or reduced statistical power to detect an interaction. The interaction of gender and age in the HIV-infected group in our study is a novel finding. This interaction may be related

to effects of decreased circulating estrogens with increasing age and subsequent inflammation. In physiological ageing, low levels of estrogens may be associated with increased levels of proinflammatory cytokines that have been linked to sarcopenia.²⁸ This effect may be exacerbated or modulated by HIV infection or ART. In resource-constrained settings, it is possible that women are nutritionally deficient compared with men, exacerbated by multiple pregnancies.

In sub-Saharan Africa, the proportion of elderly people infected with HIV is increasing.¹³ In 2007, approximately 3 million people aged 50 years or older were living with HIV in sub-Saharan Africa, comprising 14% of the adult HIV population.³⁰ With 5.1 million people in sub-Saharan Africa having started ART by the end of 2010, the number of people aged 50 years or older living with HIV will inevitably continue to increase.³¹ These evolving demographics may necessitate a shift from treatment of primarily OIs toward management of non-AIDS-related conditions. Furthermore, if premature ageing is scientifically validated, HIV-infected patients >50 years of age or older may come to be considered “old.”³² Chronic age-related disease within African HIV-infected populations is likely to place a significant burden on health care budgets and human resources. The high prevalence of HIV-related frailty also has additional economic implications. Those who are frail are less likely to be economically productive and more likely to need assistance from families and welfare grants. Interventions to improve frailty may be simple such as progressive resistance exercises. This intervention improved the strength of HIV-infected adults in Brazil.³³ However, given the multifactorial aetiology of frailty, other systemic mechanisms may also require intervention.

A key strength of this study is the inclusion of an age/gender-matched control group with a similar sociodemographic profile to the HIV-infected individuals. The hypothesis of premature ageing in HIV has received criticism primarily due to limitations in characterization of participants, in particular the possibility of differential exposure to potential risk factors between HIV-infected and HIV-uninfected populations.^{32,34,35} For example, in the US Veterans Aging Cohort, HIV-infected veterans were more likely to have a history of substance misuse compared with age- and sex-matched uninfected veterans.³⁶ The differential exposures to risk factors between HIV-infected and HIV-uninfected populations and residual confounding could result in an apparent increased risk of age-related outcomes. By recruiting from the same community, we aimed to reduce the likelihood of differential risk exposure. Compared with HIV-seronegative individuals, the HIV-infected group in this study tended to be more affluent (likely reflecting receipt of welfare grants) and also more educated as has been observed elsewhere in South Africa.³⁷ HIV remained strongly associated with frailty even after adjustment for socioeconomic factors.

This study has some limitations. We studied a frailty-like phenotype rather than the previously defined and validated frailty phenotype.¹⁷ However, this modified phenotype is comparable to phenotypes used in other studies of HIV-related frailty.^{9,10,12} The frailty phenotype within the context of HIV has not been fully established or validated to date. The main limitation of the frailty criteria relates to

their subjective nature. Grip strength and walking time measured at the time of data collection may not necessarily represent the participant’s overall ability, and a longitudinal evaluation of these parameters would be optimal. Similarly, reports of exhaustion relied on self-report. The original phenotype used a weighted score of kilocalories expended to assess low physical activity. This may be difficult to ascertain in resource-limited settings, and the approximate adopted by Onen et al¹⁰ may be more useful in these environments. Although we relied on self-report of weight loss, validation of a proportion of the reports was favorable and no differential misclassification between groups was observed.

The study design means that a causal relationship between HIV and frailty cannot be concluded, nor can a temporal relationship be established. We are unable to infer whether HIV infection or ART is primarily responsible for the strong association with frailty, partly due to a low proportion of ART-naive participants (12.9%). Often such patients are more acutely ill and may be less willing or able to take part in research studies. In the Multicenter AIDS Cohort Study cohort, the presence of frailty before ART initiation was an independent predictor of the development of AIDS or death despite ART.³⁸ Thus, frailty status may assist in establishing risk of morbidity and mortality. In the present study, we were unable to assess whether a diagnosis of frailty was related to outcomes typically associated with the syndrome in older adults, such as falls, hospitalization, and death. To fully assess if individuals in sub-Saharan Africa meeting the definition of frailty are at increased risk of these outcomes will require longitudinal studies.

In conclusion, HIV is an important predictor of frailty in this African population. Sub-Saharan Africa is undergoing significant HIV-related demographic changes, leading to an ageing HIV-infected population. Chronic age-related conditions will impact this population and HIV-related premature ageing will likely compound this disease burden. Early initiation of ART at higher CD4 counts may maintain CD4 counts at higher levels and protect against development of the frailty phenotype. As access to ART expands, and patients continue to age and live with HIV infection, longitudinal studies are needed to assess the evolution of frailty within HIV-infected populations and its impact on morbidity and mortality.

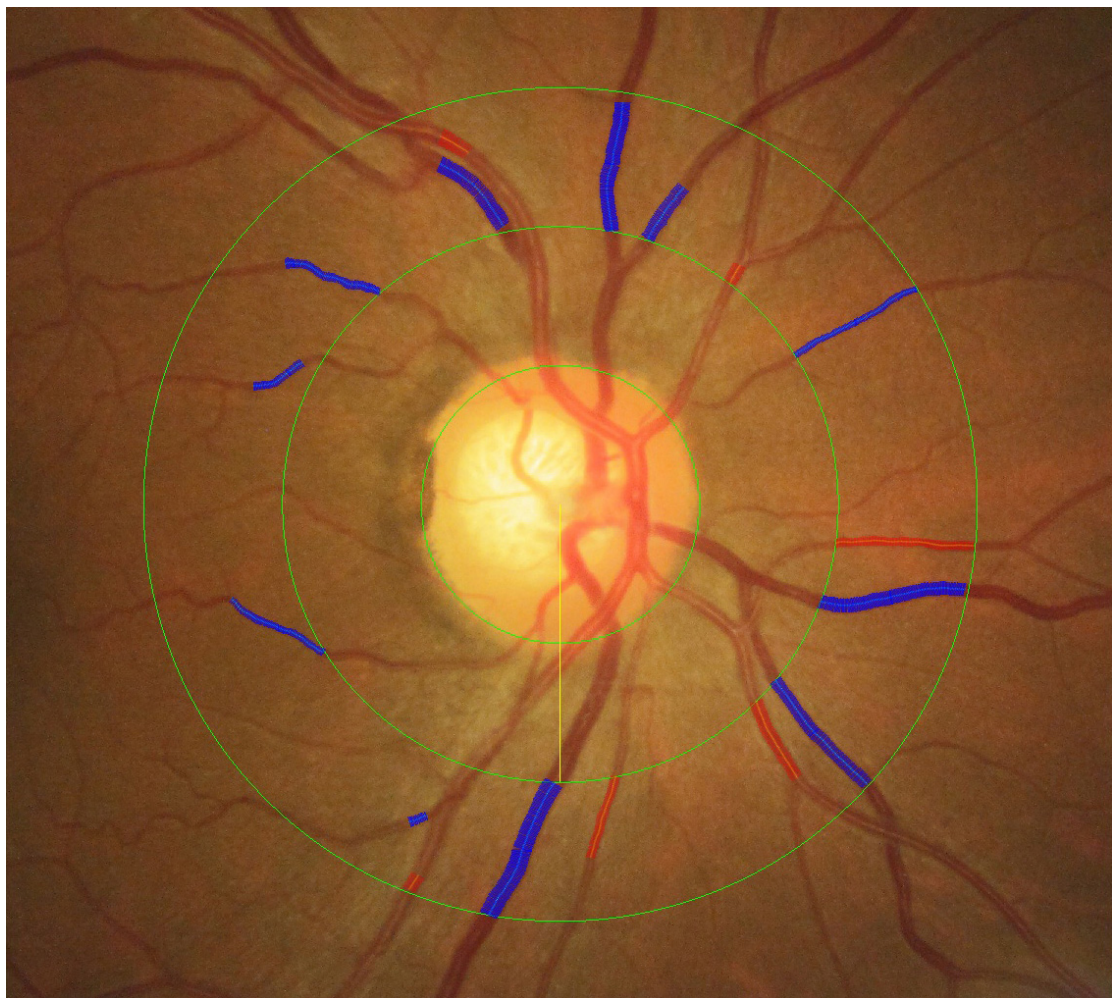
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Chapter 7

Retinal arterioles narrow with increasing duration of anti-retroviral therapy in HIV infection: a novel estimator of vascular risk in HIV?



Fundus photograph with IVAN retinal software superimposed to measure retinal vascular calibre

Research paper evaluating the relationship of retinal vessel calibre with clinical and demographic characteristics in HIV-infected and HIV-seronegative individuals

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **PLOS ONE**
 - 1.2. When was the work published? **December 2012**
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review? **Yes**
 - 1.4. Have you retained the copyright for the work? **Yes –under Creative Commons License**
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work **See Appendix 7**
2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **N/A**
 - 2.2. List the paper's authors in the intended authorship order
 - 2.3. Stage of publication – Not yet submitted/Submitted/Undergoing revision from peer reviewers' comments/In press
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments. I took all the fundus photographs in the study, and was responsible for data collection. Leris D'Costa assisted in measurement of retinal vessel calibre from fundus photographs. I performed statistical analyses with some advice from Helen Weiss. Tien Wong provided the IVAN retinal software. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



Retinal Arterioles Narrow with Increasing Duration of Anti-Retroviral Therapy in HIV Infection: A Novel Estimator of Vascular Risk in HIV?

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Abstract

Objectives: HIV infection is associated with an increased risk of age-related morbidity mediated by immune dysfunction, atherosclerosis and inflammation. Changes in retinal vessel calibre may reflect cumulative structural damage arising from these mechanisms. The relationship of retinal vessel calibre with clinical and demographic characteristics was investigated in a population of HIV-infected individuals in South Africa.

Methods: Case-control study of 491 adults ≥ 30 years, composed of 242 HIV-infected adults and 249 age- and gender-matched HIV-negative controls. Retinal vessel calibre was measured using computer-assisted techniques to determine mean arteriolar and venular diameters of each eye.

Results: The median age was 40 years (IQR: 35–48 years). Among HIV-infected adults, 87.1% were receiving highly active antiretroviral therapy (HAART) (median duration, 58 months), their median CD4 count was 468 cells/ μ L, and 84.3% had undetectable plasma viral load. Unadjusted mean retinal arteriolar diameters were $163.67 \pm 17.69 \mu$ m in cases and $161.34 \pm 17.38 \mu$ m in controls ($p=0.15$). Unadjusted mean venular diameters were $267.77 \pm 18.21 \mu$ m in cases and $270.81 \pm 18.98 \mu$ m in controls ($p=0.07$). Age modified the effect of retinal arteriolar and venular diameters in relation to HIV status, with a tendency towards narrower retinal diameters in HIV cases but not in controls. Among cases, retinal arteriolar diameters narrowed with increasing duration of HAART, independently of age (167.83μ m < 3 years of HAART vs. 158.89μ m > 6 years, p -trend = 0.02), and with a HIV viral load $> 10,000$ copies/mL while on HAART ($p=0.05$). HIV-related venular changes were not detected.

Conclusions: Narrowing of retinal arteriolar diameters is associated with HAART duration and viral load, and may reflect heightened inflammatory and pro-atherogenic states of the systemic vasculature. Measurement of retinal vascular calibre could be an innovative non-invasive method of estimating vascular risk in HIV-infected individuals.

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Introduction

HIV infection and highly active antiretroviral therapy (HAART) exacerbate a range of systemic age-related conditions such that HIV-infected patients are at increased risk of age-related non-AIDS-related morbidity and mortality compared with HIV-uninfected persons [1–3]. The emerging scenario is that of HIV population cohorts who are aging chronologically, but also likely to be undergoing accelerated physiological and immunological

senescence. Mechanisms underlying accelerated aging include increased inflammation and immune dysfunction. The microvascular circulation may reflect cumulative structural damage arising from these processes. However, current methods to investigate the micro-circulation are invasive and require specialist expertise.

The retina represents a unique location where the microvasculature can be directly and non-invasively visualised. Validated and objective quantitative measurement of retinal vessel

diameters is possible using semi-automated software applied to digitized retinal photographs [4]. This technique has been used in several population-based studies, and is established as a valid and efficient biomarker of systemic vascular disease [5–8]. Retinal vascular calibre is considered a structural marker of vascular pathology reflecting the interplay of systemic, environmental and genetic factors [9]. For example, changes in arteriolar calibre are strongly associated with chronological age, hypertension and cardiovascular disease, whereas venular calibre changes represent chronological age as well as inflammatory and cerebrovascular diseases. [10,11]. Seemingly small reductions in retinal arteriolar calibre are associated with clinically relevant changes in blood pressure, e.g., a 10-mmHg increase in systolic BP is associated with a 1.1 μm reduction in arteriolar calibre [12]. A 20 μm increase in retinal venular calibre is associated with a coronary heart disease hazard ratio of 1.16 (95% CI: 1.06–1.26) in women.

There is limited information regarding relationships between retinal vessel calibre and HIV status, particularly in the context of premature aging and risk of age-related co-morbidities such as cardiovascular disease. Furthermore, data are lacking within sub-Saharan Africa, a region where the population of older HIV-infected persons is rapidly growing as individuals initiate HAART at increasingly early stages of the disease [13]. Assessment of retinal vessel calibre can provide a non-invasive and representative model to objectively assess changes in the micro-circulation in HIV infection and with HAART.

The objective of this study was to investigate the relationship of retinal vessel calibre with clinical and demographic characteristics in a cohort of HIV-infected individuals in South Africa in comparison with a matched population of uninfected individuals. We hypothesized that retinal vascular calibre would be altered in patients with HIV, who are known to have both elevated cardiovascular risk and chronic, systemic inflammation related to HIV-related accelerated aging [14,15]. Cases and controls were recruited from neighbouring townships in Cape Town. The hypothesis of accelerated aging in HIV has received criticism primarily due to limitations in characterization of participants, and the possibility of differential exposure to potential risk factors (e.g. smoking, substance abuse) between HIV-infected and uninfected populations [16]. By recruiting from the same community, we aimed to reduce the likelihood of differential risk exposure in line with the recommendation for careful study design when investigating premature aging in HIV [17].

Methods

Ethics Statement

The study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee and the University of Cape Town Faculty of Health Sciences Ethics Committee, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study Participants

HIV-infected participants (cases) aged ≥ 30 years were enrolled from a community-based HIV treatment centre in Nyanga district of Cape Town [18,19]. All participants had a confirmed serological diagnosis of HIV and were either about to commence HAART (HAART-naïve), or were already on first-line HAART.

A control group of HIV-uninfected participants was recruited using frequency-matching by gender and 5-year age categories. Controls were enrolled from participants confirmed to be HIV-negative attending an HIV prevention trials site (Emavundleni Centre), located within the same district as the HIV treatment

centre. These two centres were chosen as individuals attending them were drawn from the same community and were therefore likely to have similar socio-demographic characteristics.

Data Collection

Socio-demographic information and medical history were obtained by questioning participants in their first language (Xhosa or English). Clinical information was obtained from medical case notes where required. Co-morbidity was defined as the concurrent presence of one or more chronic diseases or conditions including cardiovascular disease, chronic renal failure, airways disease and malignancy (both AIDS and non-AIDS defining). Cardiovascular diseases included myocardial infarction and cerebrovascular disease. Blood pressure (BP) was measured with a digital sphygmomanometer with a cuff of appropriate size. Mean arterial blood pressure (MABP) was defined as two-thirds of the diastolic plus one-third of the systolic BP. Hypertension was defined as a systolic BP of 140mmHg or higher, diastolic BP of 90mmHg or higher, or the combination of self-reported high BP diagnosis and the use of anti-hypertensive medications [20]. Body mass index (BMI) was defined as weight (in kilograms)/height². HIV infection characteristics including duration of HAART, type of HAART regimen, nadir and current CD4 count and viral load (VL) were available from the clinic database.

Retinal Vessel Measurement

All participants had stereoscopic 30 degree colour retinal photographs taken of both eyes under pharmacological pupil dilation with a fundus camera (model CF-2; Canon Inc., Tokyo, Japan). Images were centred on the optic disc. Vessel calibre indices were determined in a semi-automated manner using the IVAN computer program (Singapore Eye Research Institute, Singapore) using a standardized protocol described previously [4]. In summary, the 6 largest arterioles and venules in a ring-shaped area located between 0.5 and 1.0 disc diameter from the optic disc margin were identified (Figure 1). Computer software measured the calibre of these individual vessels, then combined them into 2 summary variables for the eye: the projected calibre size of the central retinal artery (central retinal artery equivalent [CRAE]), and the projected calibre size of the central retinal vein (central retinal vein equivalent [CRVE]), using formulas derived by Parr and Spears [21,22] and Hubbard [23], with revision by Knudtson [24]. A retinal photograph was considered ungradable if eyes had < 4 acceptable measurements of either vessel type. The inter-grader and intra-grader grading reliabilities were assessed using a random subsample of 100 photographs reviewed four weeks after the initial grading. The intra- and inter-grader intraclass correlation coefficients ranged from 0.71 to 0.93.

Data Analysis and Statistical Methods

If gradable images were available for both eyes, one eye was randomly selected for vessel calibre assessment as good correlation between eyes has been demonstrated using this software, and is considered sufficient for assessing relationships to systemic health status [25]. If there was only one gradable image, this one was used, and if neither eye had a gradable image the participant was excluded. Retinal vessel data (CRAE and CRVE) were analyzed as continuous variables. Univariable linear regression was performed to compare mean retinal arteriolar and venular diameters respectively by gender and HIV status (no; yes). Multivariable linear regression models were used to examine the relationships of retinal vessel diameters as the dependent variable with HIV status and explanatory variables (age group (30–39; 40–49; > 50 years), gender, mean arterial blood pressure; BMI,



Figure 1. Retinal vessel grading assessment. Right: fundus with automated measurements (red = arterioles, blue = venules). Left: venular diameters output.
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smoking and diastolic blood pressure). Models included an interaction term between HIV and each explanatory variable. Marginal adjusted means for retinal vessel diameters were estimated at the mean value of covariates in the model. The Wald test was used to assess statistical significance of the interaction of HIV status with each explanatory variable on retinal vessel diameter. Retinal arteriolar and venular calibre are highly correlated, and to account for potential confounding we adjusted for the fellow vessel in multivariable analyses (i.e. adjustment for arteriolar calibre in analyses of venular calibre and vice versa) [26]. All analyses were performed with Stata 12 (Stata Corp, USA).

Results

Participant Characteristics

491 participants were evaluated, of whom 242 had HIV-infection, and 249 were uninfected controls. The mean age of the HIV-infected population was 41.2 ± 0.5 years, and 42.5 ± 0.6 years in the uninfected group ($p = 0.10$). Cases had lower BMI, were less likely to be smokers, and more likely to have current or previous TB than controls (Table 1). Overall, 72.7% of cases had a history of WHO stage 3 or stage 4 defining illness. The current CD4 count among participants receiving HAART was 468 cells/ μ L (interquartile range [IQR], 325–607 cells/ μ L) and 84.0% had undetectable VL. Median treatment duration on HAART was 58 months (IQR: 34–75 months). 12.0% were HAART naïve, and had correspondingly lower CD4 counts and higher VL.

Retinal Photography and Vessel Grading

All participants underwent retinal photography. Photographs were ungradable for retinal vessel diameters in 12 individuals (2.4%), who were older than those with gradable photographs ($p = 0.001$) but otherwise had similar characteristics ($p > 0.05$ for all characteristics; data not shown). The proportion of cases and controls with ungradable photos was similar (2.48% vs. 2.41%; $p = 0.96$).

Overall Retinal Vessel Measurements

Measurements are summarised in Table 2. The following comparisons were not statistically different: arteriole diameters between cases and controls, overall ($p = 0.15$) nor by gender ($p = 0.68$ for males, $p = 0.14$ for females). However, venules were narrower in cases than controls although this did not reach statistical significance ($p = 0.07$). Male cases had significantly narrower venules ($265.72 \pm 18.04 \mu\text{m}$) than male controls ($274.55 \pm 21.66 \mu\text{m}$; $p = 0.02$) but this association was not seen in females ($p = 0.55$).

Retinal Vessel Calibre and Association with HIV and Age

In unadjusted analyses, there was a significant trend of narrower arteriolar diameters ($P\text{-trend} = 0.002$), and venular diameters ($P\text{-trend} = 0.001$) with increasing age in the HIV-infected group, also evident when stratified by gender (data not shown). This trend was not seen consistently in the control group. Table 3 reports adjusted mean vascular diameters by HIV status, stratified by other covariates. In relation to mean arteriolar diameters, age and hypertension status modified the association in relation to HIV status (P values for interaction 0.01). Mean arteriolar diameters tended to decrease with age among HIV-infected cases, and to increase among controls. Among controls, those with hypertension had wider arteriolar diameters than those without hypertension (167.97 vs. $158.28 \mu\text{m}$; $p = 0.002$), whereas among HIV cases there was little association with hypertension (164.82 vs. $163.53 \mu\text{m}$; $p = 0.69$). Mean venular diameter also decreased with age among HIV cases, but there was no association among controls ($p\text{-interaction} = 0.07$). In addition, venular diameter tended to be greater among males than females, and smokers than non-smokers among controls, but little difference was seen among cases.

Retinal Vessel Calibre in HIV-infected Participants on HAART

We investigated the associations between mean arteriolar and venular diameter and clinical/demographic factors in participants

Table 1. Demographic characteristics of study population.

Variable	Cases (242) % (n)	Controls (249) % (n)	P-value
Age (mean±SD)	41.2±0.5	42.5±0.6	0.10
Age (years) by group			
30–39	49.2 (119)	47.0 (117)	0.54
40–49	33.5 (81)	31.7 (79)	
>50	17.3 (42)	21.3 (53)	
Male gender	25.6 (62)	24.1 (60)	0.70
Hypertension status			
Hypertensive	27.3 (66)	29.3 (73)	0.62
Mean arterial blood pressure	94.37±14.64	95.53±13.90	0.37
BMI (kg/m ²)	27.8±6.5	31.5±8.8	<0.0001
Co-morbidity			
None	71.1 (172)	69.5 (173)	
One or more	28.9 (70)	30.5 (76)	0.70
Smoking status			
Smoker	15.3 (37)	27.3 (68)	0.001
TB status			
No history	30.6 (74)	88.0 (219)	<0.0001
Current	4.1 (10)	0.4 (1)	
Previous	64.3 (158)	11.7 (29)	
HIV characteristics (n = 242) (% n or median (IQR))			
WHO stage			
1/2	27.3 (66)		
3/4	72.7 (176)		
HAART naïve	12.0 (29)		
CD4 count in HAART naïve group (n = 29)	170 (84–201)		
Log ₁₀ VL* in HAART naïve group (n = 19)	4.81 (4.11–5.14)		
Current CD4 count in HAART group	468 (325–607)		
Nadir CD4 count in HAART group	127 (76–171)		
% with detectable VL in HAART group	16.0 (34)		
Peak Log ₁₀ VL in HAART group	4.56 (3.84–4.98)		
Duration of HAART, months	58 (34–75)		
HAART Regimen			
NNRTI-based*	59.6 (127)		
Other	40.4 (86)		

*VL = HIV RNA Viral load; NNRTI - non-nucleoside reverse transcriptase inhibitor;
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on HAART (Table 4). There was a trend of increasing age being associated with narrowing of retinal arterioles (P-trend = 0.08). A

longer duration of HAART was associated with narrowing of arterioles. Mean arteriolar diameter ranged from 167.83 µm in

Table 2. Retinal vessel calibre in study population.

	Mean arteriolar diameter			Mean venular diameter		
	HIV+ cases	Uninfected controls	P-value	HIV+ cases	Uninfected controls	P-value
Overall	163.67±17.69	161.34±17.38	0.15	267.77±18.21	270.81±18.98	0.07
Males	162.00±17.68	160.53±20.48	0.68	265.72±18.04	274.55±21.66	0.02
Females	164.24±17.71	161.60±16.33	0.14	268.47±18.27	269.62±17.94	0.55

Measurements are in µm and represent mean ± standard deviation.
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Table 3. Retinal Arteriolar and Venular Diameters in Relationship to HIV status, Stratified by Potential Effect Modifiers*.

	Mean arteriolar diameter μm (SE)			Mean venular diameter μm (SE)		
	HIV-infected (n = 236)	Controls (n = 243)	P-value for interaction	HIV-infected (n = 236)	Controls (n = 243)	P-value for interaction
Overall	163.95 (1.14)	161.08 (1.12)		268.17 (1.22)	270.43 (1.20)	
Age group						
30–39	165.86 (1.64)	161.27 (1.60)	0.01	271.34 (1.77)	269.49 (1.73)	0.07
40–49	163.47 (1.93)	157.94 (1.94)		265.59 (2.07)	271.43 (2.10)	
>50	159.13 (2.72)	166.24 (2.55)		264.45 (2.92)	270.96 (2.75)	
<i>P</i> -value	0.11	0.03		0.05	0.76	
Sex						
Male	161.02 (2.41)	158.29 (2.56)	0.22	266.68 (2.56)	276.13 (2.72)	0.02
Female	164.92 (1.34)	162.00 (1.36)		268.73 (1.44)	268.54 (1.44)	
<i>P</i> -value	0.17	0.23		0.50	0.02	
Cigarette use						
No	163.73 (1.24)	160.21 (1.34)	0.16	268.46 (1.32)	268.69 (1.44)	0.03
Yes	164.20 (3.09)	163.93 (2.25)		265.47 (3.29)	275.69 (2.38)	
<i>P</i> -value	0.89	0.17		0.41	0.01	
Hypertension status						
No	163.53 (1.45)	158.28 (1.42)	0.01	270.21 (1.57)	271.86 (1.54)	0.38
Yes	164.82 (2.60)	167.97 (2.46)		262.94 (2.78)	266.70 (2.66)	
<i>P</i> -value	0.69	0.002		0.04	0.12	
BMI						
<20	166.73 (3.71)	166.06 (4.36)	0.13	262.20 (3.96)	274.52 (4.66)	0.77
20–24.9	166.48 (2.20)	159.24 (2.75)		266.10 (2.36)	267.22 (2.94)	
25–29.9	160.77 (2.04)	160.77 (2.43)		266.56 (2.19)	270.04 (2.60)	
>30	163.95 (2.00)	161.18 (1.55)		271.78 (2.14)	271.93 (1.66)	
<i>P</i> -value	0.24	0.59		0.11	0.41	

Adjusted for gender, age, hypertension status, smoking status, BMI, diastolic BP and associate vessel diameter (i.e. CRVE for CRAE analyses, and CRAE for CRVE analyses), categorised as shown in the Table.

CRAE/CRVE = Central retinal artery equivalent/central retinal vein equivalent.

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those with less than 3 years duration of HAART to 158.89 μm with more than 6 years of treatment (*P*-trend = 0.02). A higher current viral load while on HAART (>10,000 copies/ml) was associated with narrower arterioles (*p* = 0.05). There was an association of narrowed arterioles with hypertension (*p* = 0.03) in this group, however this association was not apparent after adjustment for HIV-related factors (data not shown). Venular diameters narrowed with increasing age (*P*-trend = 0.02). A higher current viral load was associated with wider venular diameters in the unadjusted estimates (266.36 μm for VL <10, 000 copies/ml vs. 279.10 μm for VL >10,000 copies/ml (*p* = 0.02). The trend of wider venules with higher viral load was apparent in the fully adjusted model, but did not reach statistical significance (*p* = 0.20).

Discussion

We assessed retinal vessel calibre in HIV-infected individuals in South Africa. This study provides clear evidence that retinal arteriolar diameters narrow with increasing duration of HAART and with higher HIV viral load, independently of age. The excess age-related morbidity demonstrated in HIV-infected individuals has a significant vascular component including cardiovascular, renal and cerebrovascular disease, and retinal vessel calibre

measurement has been demonstrated as a strong biomarker of systemic vascular disease.

We found an 8.9 μm decrease in arteriolar diameter in participants who had been on HAART for >6 years after adjustment for age and HIV-related factors. Our findings are in alignment with data from the Longitudinal Studies of the Ocular Complications of AIDS (LSOCA) [27], in particular, the association of narrower retinal arteriolar diameter with exposure to HAART. In our study population we additionally demonstrated that retinal arteriolar diameter decreases with increasing duration of HAART. The association of retinal arteriolar calibre with cardiovascular risk is well documented. Retinal arteriolar diameter (CRAE) is independently associated with increased carotid intima thickness [12], and with higher cardiovascular mortality risk in older persons [5,28]. An important cause of premature morbidity and mortality in HIV-infected individuals is from cardiovascular complications [29]. HAART-treated patients have a greater prevalence of atherosclerosis and vascular dysfunction than age-matched uninfected adults [30]. Enhanced endothelial dysfunction (measured by flow-mediated dilation) and increased carotid intima media thickness have been demonstrated in HIV cases compared to controls, despite antiretroviral therapy and adjustment for traditional CVD risk factors [31–35]. It is plausible that narrower

Table 4. Retinal vessel diameters in association to HIV-related factors in participants on HAART.

	N	Arteriolar diameter (n = 207)		Venular diameter (n = 207)	
		Mean (SE), μm	P	Mean (SE), μm	P
Age group (years)					
30–39	99	164.81 (1.87)		269.93 (1.91)	
40–49	73	163.40 (2.17)	0.08*	266.49 (2.21)	0.02*
>50	35	157.31(3.20)		260.37 (3.26)	
Sex					
Male	50	160.67 (3.05)		265.78 (3.12)	
Female	157	163.80 (1.49)	0.37	267.52 (1.53)	0.64
Hypertension status					
No	148	161.58 (1.93)		268.31 (1.98)	
Yes	59	166.72 (4.00)	0.35	264.06 (4.08)	0.42
HAART duration, months					
0–36	60	167.83 (2.56)		269.70 (2.63)	
37–72	86	162.65 (1.97)		265.91 (2.01)	
>73	61	158.89 (2.38)	0.02*	266.22 (2.45)	0.57
Current CD4 count					
<500 cells/ μL	120	163.04 (1.66)		266.80 (1.70)	
>501 cells/ μL	87	163.05 (1.99)	0.98	267.51 (2.03)	0.78
Nadir CD4 count					
<200 cells/ μL	184	162.85 (1.29)		266.37 (1.31)	
>201 cells/ μL	23	164.58 (3.94)	0.67	272.92 (4.01)	0.13
Current HIV viral load					
<10,000 copies/ml	195	163.71 (1.25)		266.65 (1.27)	
>10,000 copies/ml	12	152.21 (5.64)	0.05	274.40 (5.79)	0.20
Peak HIV viral load					
<10,000 copies/ml	59	164.32 (2.37)		268.59 (2.42)	
>10,000 copies/ml	148	162.54 (1.45)	0.50	266.51 (1.48)	0.46

Adjusted for age, gender, hypertension status, mean arterial blood pressure (MABP), smoking status, BMI, diastolic BP, associate vessel diameter (i.e. CRAE for CRAE analyses, and CRAE for CRVE analyses), current CD4 count and nadir CD4 count, current VL and peak VL, HAART duration, HAART regimen, WHO clinical stage (1/2 or 3/4) and TB status (current or past history vs. no history).

*P-value for test of trend.

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retinal arteriolar diameter is related to excess cardiovascular risk in patients on HAART. The risk of cardiovascular disease appears to be greatest with protease inhibitors (PIs) compared to non-nucleoside reverse transcriptase inhibitors (NNRTIs) [36,37]. The majority of our study population were treated with NNRTIs, and we postulate that the magnitude of arteriolar narrowing might be greater in populations where treatment is PI-based.

Higher viral load while on HAART (>10,000 copies/mL) was associated with wider venular calibre in unadjusted estimates; this trend remained in an adjusted model, but did not achieve statistical significance. Endothelial dysfunction is associated with larger retinal venules independent of traditional cardiovascular risk factors [38]. Larger venular calibre is also associated with higher levels of inflammatory markers such as interleukin-6 [39,40]. We postulate that in our HIV-infected population higher HIV viral loads may cause inflammation and/or endothelial dysfunction manifest as retinal venular dilation. The association of narrow venular diameter with increasing age was stronger in an adjusted model. This may reflect lack of statistical power, or possibly that the 'aging' phenotype plays a larger role in determining venular calibre in HIV. Narrow arteriolar calibre is

also associated with inflammation in diabetes possibly explaining the finding of narrower CRAE with higher viral load [11].

The strong association between increasing age and narrowed retinal vessels has been demonstrated in several study populations [41–43]. This association was demonstrated in our cohort of HIV-infected individuals, and in a US HIV population [27]. We hypothesized that retinal vascular changes typically occurring in older populations might occur earlier in life in HIV-infected individuals. For venular diameters this appears to be consistent, however, this trend was not apparent in retinal arterioles. The interaction of age with HIV status in determining retinal arteriolar diameter is novel and biologically difficult to explain. Graders were masked to the HIV status of the participants, and misgrading would have caused random misclassification. There may be residual confounding and bias accounting for this finding, or this scenario could have occurred by chance. The effect of age in relation to HIV status, and possible effect modification warrants further investigation.

The strengths of this study include the high proportion of gradable digital retinal photographs, and use of a well-established, standardized computer-based technique to measure retinal vascu-

lar calibre. The study design also permitted inclusion of an age/gender matched control group with a similar socio-demographic profile as the cases. The hypothesis of accelerated aging in HIV has received criticism primarily due to limitations in characterization of participants, and the possibility of differential exposure to potential risk factors between HIV-infected and uninfected populations [16,44,45]. By recruiting from the same community, we aimed to reduce the likelihood of differential risk exposure.

Despite overall high reproducibility using computer-assisted methods, many factors may affect vessel measurement, some of which are inherent. For example, slight changes in vessel diameter with the cardiac cycle (due to pulsatility) may result in variation in vessel measurements [46] but fluctuations are small and random [9], causing non-differential misclassification. Measurement of vessel diameters from colour retinal photographs may underestimate true vascular width because only the red blood cell column is measured, and not the peripheral plasma cuff. Other factors relate to the population studied e.g. clarity of the ocular media and hence photographs, and the presence of greater retinal pigment, as in African retinas, may overestimate retinal vessel diameter [47]. Our study design does not permit conclusions about the temporal relationship between changes in retinal calibre and subsequent risk of morbidity (e.g. cardiovascular events) or mortality from these data. In addition, we cannot make any inference about whether HIV infection or HAART is primarily responsible for the changes in vascular calibre due to the low proportion of HAART-naïve individuals recruited. Finally, as both case and control populations are of African ancestry these findings may not be generalisable to other ethnic groups.

We present novel data on retinal vascular calibre in HIV. It is reassuring that our findings are in alignment with a US HIV cohort [27]. However, compared to US HIV populations, it is likely that African HIV populations will have experienced a shorter duration of HAART and initiated treatment at lower CD4 counts. The atherogenic, inflammatory and aging effects of HIV and HAART and related changes in retinal vascular calibre may

thus follow a different trajectory, with longitudinal data needed to assess this definitively. It is also unclear whether HIV infection or treatment with HAART is primarily responsible for changes in vascular calibre. In addition, epigenetic and genetic variation may contribute to an individual's susceptibility to non-HIV age-related morbidity and to vascular structural changes.

The challenge of managing excess age-related morbidity in HIV-infected individuals will increase as HIV populations live longer and HAART coverage expands. Stratification of vascular risk among HIV-infected individuals, particularly cardiovascular risk, and implementing preventive strategies will be important priorities for patient management. Cardiovascular risk assessment tools already exist such as the Framingham Risk Scores [48], however, specific tools tailored to HIV-infected populations are necessary [49]. Measurement of retinal vessel calibre is validated, non-invasive, and can be performed quickly by non-clinical personnel. Our data support the hypothesis that retinal vascular calibre changes occur in HIV infection, reflecting systemic vascular pathology. Longitudinal studies are needed to confirm this hypothesis as well as validation studies to explore the role of retinal vessel measurement as a tool in HIV-related vascular risk estimation.

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Author Contributions

Conceived and designed the experiments: SP CG SDL TP. Performed the experiments: SP LD. Analyzed the data: SP HW LD. Contributed reagents/materials/analysis tools: TW CC. Wrote the paper: SP HW SDL TP CG.

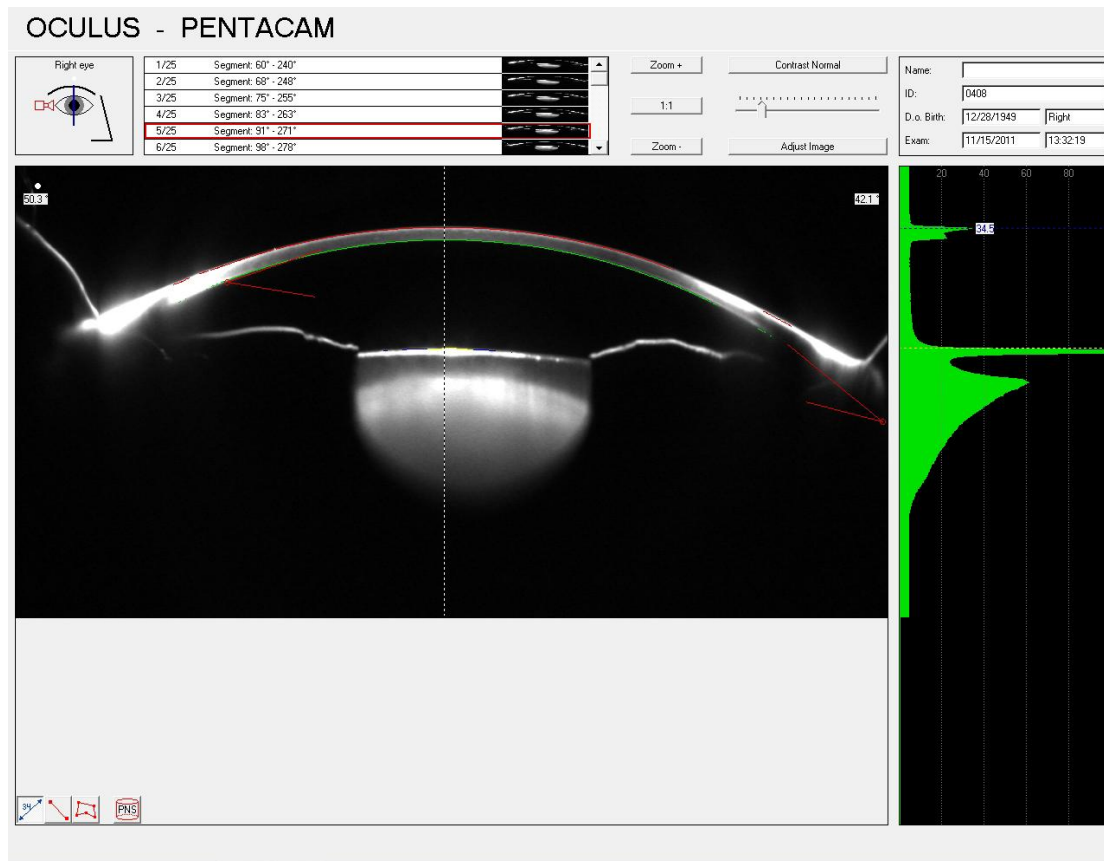
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Chapter 8

Increased ocular lens density in HIV-infected individuals with low nadir CD4 counts in South Africa: evidence of accelerated aging



Pentacam evaluation of lens density – this amount of lens opacity would be clinically apparent as cataract

Research paper investigating the relationship of lens density measurements with clinical and demographic characteristics in HIV-infected individuals in comparison with HIV-seronegative individuals

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work
2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **Journal of Acquired Immunodeficiency Syndromes (JAIDS)**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, SD Lawn, HA Weiss, C Cook, LG Bekker, CE Gilbert
 - 2.3. Stage of publication — ~~Not yet submitted~~/Submitted/**Undergoing revision from peer reviewers' comments**/In press
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments and conducted the data collection. I performed all Pentacam imaging and clinical examinations. I performed the statistical analyses with advice from Helen Weiss. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



TITLE PAGE

Increased ocular lens density in HIV-infected individuals with low nadir

CD4 counts in South Africa: evidence of accelerated aging

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Abstract

Background

HIV infection is thought to be associated with an increased risk of age-related morbidity and premature aging. Lens density increases with age and may function as a biomarker of aging. We investigated the relationship of lens density measurements with clinical and demographic characteristics in HIV-infected individuals in comparison with a matched population of HIV-seronegative individuals.

Methods:

Case-control study of 490 adults aged ≥ 30 years, composed of 242 HIV-infected adults and 248 age- and gender-matched HIV-seronegative individuals. Lens density was assessed using lens densitometry (Pentacam) imaging. Measurements were divided into quartiles and comparison of HIV status and HIV-related factors was assessed by multivariate and multinomial logistic regression.

Results:

The mean age was 41.2 years in HIV-infected adults and 42.3 years in HIV-seronegative individuals ($p=0.14$). Of the HIV-infected adults, 88% were receiving antiretroviral therapy (ART) (median duration, 58 months), and within this group their median CD4 count was 468 cells/ μL , and 84% had undetectable viral load. Although adjusted lens densities were similar by HIV serostatus, participants on ART and who had nadir CD4 counts <200 cells/ μL had a higher risk of high lens density compared to HIV-seronegative individuals ($p\text{-trend}=0.04$). Lens density was weakly associated with detectable HIV viremia despite ART, but not with current CD4 count.

Conclusions:

HIV-infected individuals on ART with nadir CD4 counts <200 cells/ μ L had increased risk of higher lens density. Lens density may represent a novel biomarker of aging, providing insight into accelerated aging trajectories in HIV infection.

Word count: 242

MAIN TEXT

Introduction:

Antiretroviral therapy (ART) has reduced mortality among HIV-infected people, largely due to prevention of AIDS-related events, but also due to a decrease in non-AIDS related events and deaths.^{1,2} However, it is now of concern that ART cohorts are aging. In the USA, for example, it is estimated that by 2015 more than 50% of the HIV-infected population will be over 50 years of age.³ This trend is becoming apparent in sub-Saharan Africa where there is an expanding cohort of HIV-infected individuals who are aged over 50.^{4,5} Patients receiving ART are thought to be at increased risk of age-related non-AIDS morbidity and mortality compared with HIV-seronegative persons.^{6,7} Several of these conditions are classically associated with the normal aging process but occur at an earlier age in HIV-infected persons compared to age-matched HIV-seronegative individuals.⁸ Thus, HIV infection may be associated with accelerated senescence, and the increased risk of systemic age-related morbidities in HIV-infected people has frequently been found to be associated with nadir CD4 count and ART duration.⁹⁻¹²

Cataracts are opacities of the intraocular lens and the prevalence of lens opacities increases exponentially with age.¹³ They are a leading cause of blindness and visual impairment worldwide.¹⁴ Individuals with cataract have an increased mortality risk than those without, even after adjusting for known confounders.^{15,16} The human lens is considered an ideal tissue for studying macromolecular aging as biochemical mechanisms in lens proteins may reflect aging processes elsewhere in the body.¹⁷⁻²⁰ In the context of lens opacities and HIV, two possible scenarios exist. Firstly, although the prevalence of HIV-related opportunistic ocular infections (such as

cytomegalovirus retinitis) has decreased,²¹ the introduction and scale-up of ART has led to immune-recovery phenomena.²² This may manifest in the eye as ‘immune recovery uveitis’ (IRU). It is characterized by ocular inflammation following ART initiation, and can lead to cataract formation.²³ Secondly, if premature aging does occur in HIV-infected individuals, an increased risk of ocular age-related conditions such as cataract might be expected. To date, there is minimal information about age-related eye conditions and the possibility of accelerated aging in HIV-infected patients, particularly within sub-Saharan Africa.

The Pentacam (Oculus, Germany) is a 360-degree rotating non-contact camera that provides a rapid and precise three-dimensional view of the lens, cornea and anterior chamber.²⁴ The Pentacam uses a blue light-emitting diode (wavelength 475nm) to image the anterior eye segment, capturing 25 single-slit images in 2 seconds while rotating around the eye from 0 to 180 degrees. The instrument acquires data points that provide a representative image of the whole lens.²⁵ The Pentacam contains integral software that can automatically quantify lens ‘density’ by measuring the light-scattering intensity of the lens layers. This provides a form of objective lens opacity (cataract) grading. The objective of the study was to assess differences in lens density using the Pentacam in a cohort of HIV-infected individuals in comparison to an age- and gender-matched HIV-seronegative group. Our study hypotheses were that (a) HIV-infected individuals will have a greater lens density compared to gender- and age-matched HIV-seronegative individuals and (b) low nadir CD4 count at ART initiation is associated with an increased risk of higher lens density. Our study was conducted in Cape Town, South Africa, with recruitment of cases and controls from the same communities to reduce the likelihood of

differential risk exposure in line with the recommendation for careful study design when investigating premature aging in HIV.²⁶

Methods:

Study participants:

HIV-infected participants aged ≥ 30 years were enrolled from a community-based HIV treatment centre in Nyanga district in Cape Town.^{27,28} Participant recruitment has been reported in detail elsewhere.²⁹ In brief, all participants had a confirmed serological diagnosis of HIV and were either about to commence ART (ART-naïve), or were already on first-line ART. A control group of HIV-seronegative participants were frequency-matched to cases by gender and 5-year age categories. Participants who reported ocular symptoms (other than relating to decreased vision), or were found to have active ocular pathology (excluding lens opacities) at study enrolment were excluded from the study to reduce any potential biases, and were referred for treatment where necessary. In addition, participants with diabetes were not recruited due to the possible confounding effect of diabetes on premature aging and increased risk of lens opacities.

Pilot data obtained from the HIV-infected population was used to inform sample size calculations, as well as reference to published data relating to cataract prevalence in Africa.³⁰ A sample size of 480 (240 HIV-infected and 240 HIV-seronegative individuals) provided 80% power at a 95% significance level to detect a difference in moderate lens density (i.e. clinically detectable cataract, with visual acuity $<20/40$) between the two groups.

Data collection:

Data collected included factors known to affect aging and age-related parameters (e.g. smoking and work location (indoor/outdoor) as a proxy for UV exposure). Clinical information was obtained from medical case notes where required. All participants underwent a full ophthalmic examination including measurement of visual acuity, evaluation by slit lamp microscopy (for anterior segment structures e.g. cornea, anterior chamber) and indirect ophthalmoscopy (for retina and vitreous). Participants also underwent refraction in order to assess their refractive state (i.e. emmetropia [no refractive error], myopia [short-sightedness] or hypermetropia [long-sightedness] as this can contribute to the development of lens opacities.

Lens density measurement:

Following pupil dilatation with 1% tropicamide, two lens density measurements were made on each eye, and the mean value calculated. The instrument automatically calculated the quality and reliability of a captured image. If an image was found to be of poor quality (i.e. not 'OK' on the image quality specification), the measurement was repeated. Reproducibility of the lens density evaluation in two scans was performed for 50 eyes. Lens densitometry output values were extracted from the image captures in a masked fashion. Image section 90-270 degrees was used for the right eye and image section 270-90 degrees was used for the left eye.²⁵ All of the densitometry metrics available from the scan (linear, peak and 3D) were analysed as they reflect different parameters of the lens. Figure 1 shows an example of the metrics. For the linear metric, a line was drawn through the visual axis and a mean lens densitometry value given. The peak metric indicated the point at which lens densitometry was greatest on the lens densitogram. For the 3D metric, a fixed,

circular 3.0 mm area of the lens was selected and a mean lens densitometry value given.²⁵ The lens density output was presented on a continuous standardized scale from 0 (transparent) to 100 (fully opaque). Further examples of Pentacam images obtained and the relevant metrics are presented as Supplementary Figures S1-S5.

The study was approved by the Ethics Committees of the London School of Hygiene and Tropical Medicine and the University of Cape Town Faculty of Health Sciences, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants

Statistical analyses:

One eye was randomly selected for analysis. Where an eye was not available, for example due to trauma or corneal opacity, the contralateral eye was used. Although the Pentacam has been validated against clinical grading scores, there are no standard reference scores as to what might constitute a clinically significant degree of lens opacity, thus lens density measurements were divided into quartiles defined by control group measurements. The association of HIV status with lens density was assessed in univariable and multivariable logistic regression models adjusted for risk factors and *a priori* confounders (UV exposure, smoking). Multinomial logistic regression was used to assess the association of lens density with 4 different outcome groups (e.g. uninfected, HIV-infected on ART with nadir CD4 count <200 cells/ μ L, HIV-infected on ART with nadir CD4 count \geq 200 cells/ μ L, HIV-infected ART naive). In analyses restricted to HIV-infected participants on ART, we assessed relationships between quartiles of lens density and HIV-related variables of interest

including duration of ART, nadir CD4 count, current CD4 count and viral load. All analyses were performed with Stata 11 (Stata Corp, USA).

Results

Participant characteristics:

Characteristics of the 242 HIV-infected individuals and 248 age/gender frequency matched HIV-seronegative individuals are shown in Table 1. Overall, the groups were well matched. The HIV-infected group tended to have a greater income (attributable to a government health-related grant), less reported consumption of alcohol ($p=0.002$) and fewer cigarettes ($p=0.001$). They were also most likely to report a previous eye condition than HIV-negative controls ($p=0.01$). None of the participants had undergone ophthalmic surgery, and in particular none had cataract surgery for either eye or had had clinically active uveitis (a risk factor for cataract). Visual acuities were similar in the two groups ($p=0.62$). The majority of the HIV-infected group were on already on ART, 12% were treatment naïve. The median duration on ART was 58.5 months, [interquartile range (IQR); 34-75.8], and 84% of participants had an undetectable plasma viral load. The median current CD4 count in this group was 468 cells/ μL (IQR: 325-607)

Lens density measurements:

Lens density values of sufficient quality were available for 484 (98.0%) of the 490 study participants. None of the participants had high myopia or hypermetropia. Acquisition of sub-optimal densitometry images was mainly related to participant movement and rapid blinking during image capture (preventing acquisition of all 25 slit images). Participants who did not have good quality lens density images had

similar characteristics to those who did (P-value for all characteristics >0.10, data not shown).

Unadjusted median lens density values were similar between HIV-infected individuals and HIV-seronegative individuals. The median linear value was 9.8 (IQR: 9.2-10.0) in HIV-infected individuals and 9.8 (9.6-9.9) in HIV-seronegative individuals ($p=0.75$). Corresponding peak values in the two groups were 17.9 (15.4-19.9) and 18.0 (15.4-20.5) ($p=0.43$) and 3D values were 9.7 (9.1-10.6) and 9.8 (9.1-10.9) ($p=0.29$), respectively. Table S1 compares lens density between the two groups – as expected, age was a strong independent predictor of high lens density in both groups ($p<0.0001$). In the HIV-seronegative group, indoor work was protective against high lens density (OR 0.35; 95%CI: 0.12-1.00; $p=0.05$) as was greater income ($p=0.06$).

Associations between lens density and HIV status (including both those on ART and ART-naïve) are shown in Table 2. After adjustment for potential confounding variables, there was a trend of a higher linear lens density associated with HIV infection (Odds ratio [OR]=1.42, 95% CI: 0.67-3.03 for highest vs lowest quartile), although this did not reach statistical significance (p -trend=0.31).

Table 3 reports adjusted ORs of HIV status with lens density metrics using multinomial logistic regression. After adjustment for potential confounding variables, participants on ART with nadir CD4 count <200 cells/ μ L were more likely to have high lens density than HIV-seronegative individuals (p -trend=0.04). In contrast, there was no significant association between lens density and HIV infected participants who were ART-naïve or on ART with nadir CD4 count \geq 200 cells/ μ L.

We next assessed associations with lens density among HIV-infected participants. Table 4 reports lens density quartiles in relation to nadir CD4 count in participants on ART. In univariable analyses there was some evidence that HIV infected participants with a nadir CD4 count below 200 cells/ μ L were more likely to have greater linear lens density than those with higher nadir CD4 counts (p-trend=0.16). This trend strengthened after adjustment for demographic and HIV-related covariates (p-trend=0.04). A similar result was seen for the 3-D value of lens density but not peak value. Duration of ART did not affect any of the three lens density metrics, neither were there any specific associations with different types of ART regimen. Current CD4 count was not related to lens density. There was a trend for uncontrolled HIV viremia (i.e. detectable VL [>50 copies/ml] despite ART) to be associated with higher lens density, however this was not significant for any of the metrics (See Supplementary Tables S2 and S3).

Discussion

We have previously demonstrated in this South African study population that HIV infection is associated with increased risk of frailty and with changes in the retinal vasculature that may reflect premature aging and increased cardiovascular risk.^{29,31} These previous studies both provided important evidence that HIV infection is associated with a clinical phenotype that is consistent with accelerated senescence. The present study further builds on these findings, demonstrating that HIV-infected individuals receiving ART and who had low nadir CD4 cell counts (<200 cells/ μ L) also have a greater risk of increased ocular lens density when compared with HIV-

seronegative individuals. This provides corroborative evidence of accelerated aging and that this may be associated with delayed initiation of ART.

As cataract is strongly related to aging, lens density might be expected to be greater in such individuals compared to age- and gender-matched HIV-seronegative individuals. We used an objective method to assess lens density rather than a clinical diagnosis of cataract. The association between low nadir CD4 counts and greater risk of high lens density persisted after adjustment for HIV-related covariates. ART duration or current CD4 count, however, did not affect lens density status.

Low nadir CD4 count has been identified as an independent predictor of several premature age-related co-morbidities in HIV-infected individuals.⁹⁻¹¹ The CD4 count at which ART is initiated is likely to be a key modifiable risk factor with regard to the excess risk of non-AIDS conditions described in observational HIV study cohorts.^{10,32} There is also potential for a reduction in non-AIDS related morbidity and mortality from earlier initiation of ART.³³ Initiating ART at higher CD4 counts may alter the 'aging trajectory', and may be an additional benefit derived from early treatment. Furthermore, improved survival of patients in African settings has been associated with CD4 counts >500 cells/ μ L following ART.³⁴ It is possible that the duration of time accumulated at low CD4 counts is associated with accrual of HIV-related 'aging damage', and thus the rate of CD4 cell decline might be an important modifiable factor in alteration of the aging trajectory. We did not detect an association between ART duration or regimen with lens density. These parameters have been associated with some premature age-related morbidities, but not others.

^{9,10,35,36} It is possible that in the eye ART may have a limited effect on lens fibres, as they are metabolically inactive.

Despite differences in lens density between HIV-infected individuals with low nadir CD4 counts and HIV-seronegative individuals, a significant difference between the two overall groups was not observed. In sub-Saharan Africa, where ART coverage is still expanding, lens density changes may not yet be detectable along the life course aging trajectory compared to HIV-seronegative individuals. Lens opacities typically occur over years, and so differences may become more marked if assessed over a longer period of time. Chronological age will be an important compounding factor in the development of lens opacities as the proportion of individuals over the age of 50 within ART cohorts continues to grow. Prospective longitudinal studies of similar study cohorts will be necessary to provide further information about the utility and validity of lens density as a biomarker of aging, particularly in relation to the possibility of accelerated aging and HIV infection. Concurrent measurement of other biomarkers of aging (e.g. telomere length) may help to validate this as a tool.

A key strength of our study is the inclusion of an age- and gender-matched control group with a similar socio-demographic profile as the HIV-infected individuals. The hypothesis of accelerated aging in HIV has received criticism primarily due to limitations in characterization of participants, particularly the possibility of differential exposure to potential risk factors between HIV-infected and uninfected populations.^{12,37,38} Since the HIV epidemic in South Africa is generalized and recruitment took place from the same community, the likelihood of differential risk exposure was minimized. Another strength is the objective assessment of lens

status. A cohort study of HIV-infected individuals in Denmark reported a cataract surgery incidence risk ratio of 1.87 (95%CI: 1.50-2.33) compared to HIV-seronegative individuals.³⁵ It is difficult to directly compare our findings due to the different outcome measures. In resource-limited settings, access to cataract surgery is often limited, thus cataract surgery as an indicator of the presence of cataract/high lens density may provide inaccurate information. Measurement of lens density may therefore be a more accurate indicator of age-related lens changes. Indeed, the phenotype of 'lens transparency' has been described as a possible biomarker of aging.³⁹

Previous assessments of lens opacity for research purposes have used clinical grading systems such as the Lens Opacities Classification System (LOCS) III.⁴⁰ However, such systems are based on subjective grading using methods such as slit-lamp evaluation and lens photography. Pentacam lens images have been validated against LOCSIII, and repeatability of measurements and correlation with clinical grading has been demonstrated.^{24,25} Lens density is also expressed as a continuous variable rather than graded steps (as in LOCSIII), which is useful for detecting small amounts of lens opacity. This was of particular importance for our study population (median age 40 years), where lens opacities would be difficult to discern clinically. We found the linear metric to be most informative. The peak metric tended to measure isolated 'spikes' of high lens density and was probably not representative of gradual age-related changes within the lens fibres.

There are some limitations to our study. Although our hypothesis relates to accelerated aging in HIV, we cannot exclude the possibility of intraocular inflammation accounting for the observed relationship of nadir CD4 count with

higher lens density. This could have arisen from immune recovery uveitis (IRU) in patients with cytomegalovirus (CMV) infection, for example. However, we did not detect any evidence of IRU and we have previously shown the prevalence of CMV retinitis to be very low within this population.⁴¹ Therefore, our current data are more likely to support the hypothesis of premature aging in line with other systemic co-morbidities.^{9,42} Reports of smoking and alcohol intake were considerably lower in HIV-infected individuals compared to HIV-seronegative individuals. This could partially explain the finding of similar lens density values between the two groups; smoking in particular is a risk factor for cataract,⁴³ thus even if accelerated aging is occurring in HIV-infected individuals, low smoking status within this group may decrease the magnitude of difference between HIV-infected individuals and HIV-seronegative individuals. It is possible HIV-infected participants may have adopted healthier lifestyle behaviours, or they may have not reported true smoking habits.

In conclusion, we used measurement of lens density as a non-invasive method of assessing biological aging. We found HIV-infected individuals on ART with low nadir CD4 counts to have an increased risk of higher lens density compared to HIV-seronegative individuals. These data support our previous findings in this study population that collectively provide evidence of HIV-related accelerated senescence.^{29,31} The present data may have implications relating to early initiation of ART to potentially avert some of these long-term but as yet unquantified complications. Premature aging may well have important socio-economic and healthcare implications for the many millions of people living with HIV long-term on ART in sub-Saharan Africa.

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TABLES (FOR MAIN MANUSCRIPT)

Table 1: Characteristics of study participants

Variable	HIV-infected (242) % (n)	HIV- seronegative (248) % (n)	P-value
Age (mean±SE)	41.2±0.5	42.3±0.6	0.14
Age, years by group			
30-39	119 (49.2)	117 (47.2)	0.60
40-49	81 (33.5)	79 (31.8)	
>50	42 (17.4)	52 (21.0)	
Male gender	25.6 (62)	24.2 (60)	0.72
Working location			
Indoors	35.1 (85)	28.2 (70)	0.10
Income			
>ZAR1000/month	42.2 (102)	31.5 (78)	0.01
Smoking status			
Smoker	15.3 (37)	27.4 (68)	0.001
Alcohol			
Yes	30.2 (73)	44.0 (109)	0.002
History of eye condition			
Yes	16.9 (41)	9.3 (23)	0.01
Visual acuity (presenting)	20/25	20/25	0.62
% with visual acuity <20/40	6.2 (15)	9.7 (24)	0.16
Clinical lens status			
Clear	76.0 (184)	71.4 (177)	0.40
Non visually-significant cataract ^a	19.4 (47)	21.8 (54)	
Visually-significant cataract	4.6 (11)	6.9 (17)	
<i>HIV-related characteristics</i>			
WHO stage			
1/2	27.3 (66)		
3/4	72.7 (176)		
HAART naïve	12.0 (29)		
CD4 count in HAART naïve group (n=29)	170 (84-201)		
Log ₁₀ VL in HAART naïve group (n=19)	4.81 (4.11-5.14)		
Current CD4 count in HAART group	468 (325-607)		
Nadir CD4 count in HAART group	127 (76-171)		
% with detectable VL in HAART group	16.0 (34)		
Peak Log ₁₀ VL in HAART group	4.56 (3.84-4.98)		
Duration of HAART, months	58 (34-75)		
HAART Regimen			
Containing AZT/3TC	59.6 (127)		
Other	40.4 (86)		

a -Visually-significant cataract defined as clinically detectable cataract and vision in worse eye <20/40

Table 2: Association of lens density with HIV

Lens density	N	HIV cases (%)	Univariate OR (95%CI)	P-value	Multivariate ^a OR (95%CI)	P-value
Linear value, all participants						
First quartile (lowest)	128	63(26.5)	1		1	
Second quartile	121	61 (25.6)	1.05 (0.64-1.72)		1.15 (0.87-1.94)	
Third quartile	118	58 (24.4)	1.00 (0.60-1.65)	0.80*	1.36 (0.68-2.48)	0.31*
Fourth quartile (highest)	117	56 (23.5)	0.95 (0.57-1.56)		1.42 (0.67-3.03)	
Peak value, all participants						
First quartile	121	59 (24.8)	1		1	
Second quartile	127	65 (27.3)	1.14 (0.69-1.87)		1.30 (0.77-2.18)	
Third quartile	123	60 (25.6)	1.03 (0.63-1.71)	0.61*	1.25 (0.73-2.15)	0.87*
Fourth quartile	113	54 (21.9)	0.90 (0.54-1.50)		1.05 (0.59-1.88)	
3-D value, all participants						
First quartile	133	71 (29.8)	1		1	
Second quartile	105	54 (22.7)	0.74 (0.45-1.21)		0.83 (0.49-1.40)	
Third quartile	120	65 (27.3)	0.96 (0.59-1.57)	0.37	1.15 (0.62-2.11)	0.31
Fourth quartile	115	48 (20.2)	0.69 (0.41-1.14)		0.79 (0.35-1.75)	

* - Refers to p-value for test of linear trend

a - Adjusted for age, gender, UV exposure status, smoking status, alcohol intake, income and history of eye condition

Table 3: Association of lens density with HIV status using multinomial logistic regression – uninfected controls represent baseline

Lens density	HIV+ on HAART, nadir CD4 count >200 cells/ μ L		HIV+ on HAART, nadir CD4 count <200 cells/ μ L		HIV+, HAART-naïve ^c	
	OR (95%CI) ^b	P-value	OR (95%CI) ^b	P-value	OR (95%CI) ^b	P-value
Linear value						
First quartile	1		1		1	
Second quartile	0.57 (0.18-1.82)		1.57 (0.89-2.77)		0.37 (0.11-1.24)	
Third quartile	0.62 (0.15-2.53)	0.16	1.91 (1.00-3.67)	0.04*	0.43 (0.12-1.52)	0.25*
Fourth quartile	0.22 (0.03-1.62)		2.16 (0.95-4.93)		0.58 (0.12-2.71)	
Peak value						
First quartile	1		1		1	
Second quartile	1.38 (0.43-4.45)		1.20 (0.66-2.09)		1.94 (0.65-5.80)	
Third quartile	1.14 (0.31-4.23)	0.71	1.22 (0.69-2.17)	0.90	1.54 (0.48-4.94)	0.91
Fourth quartile	1.39 (0.37-5.21)		1.03 (0.55-1.91)		0.96 (0.25-3.64)	
3-D value						
First quartile	1		1		1	
Second quartile	0.29 (0.06-1.15)		1.09 (0.62-1.91)		0.42 (0.13-1.31)	
Third quartile	0.63 (0.16-2.53)	0.19	1.51 (0.78-2.91)	0.67	0.41 (0.11-1.59)	0.33
Fourth quartile	0.26 (0.04-1.92)		0.99 (0.43-2.33)		0.62 (0.11-3.32)	

* - Refers to p-value for test of linear trend

b - Multivariate Odds ratios adjusted for age, gender, UV exposure status, smoking status, alcohol intake, income and history of eye condition

c -Median CD4 count in this group was 127 cells/ μ L

Table 4: Association of lens density with nadir CD4 count in HIV-infected participants on HAART

Lens density	N	HIV+ on HAART; nadir CD4 >200 cells/ μ l (%) ^d	Univariate		Multivariate ^e	
			OR (95%CI)	P-value	OR (95%CI)	P-value
Linear value						
First quartile (lowest)	50	10 (41.7)	1		1	
Second quartile	57	5 (20.8)	0.39 (0.12-1.21)	0.16	0.29 (0.07-1.22)	0.04
Third quartile	53	6 (25.0)	0.51 (0.17-1.53)		0.60 (0.10-3.67)	
Fourth quartile (highest)	49	3 (12.5)	0.26 (0.07-1.01)		0.05 (0.01-1.06)	
Peak value						
First quartile	53	6 (25.0)	1		1	
Second quartile	55	7 (29.2)	1.14 (0.36-3.65)	0.95	1.19 (0.29-4.91)	0.60
Third quartile	52	4 (16.7)	0.65 (0.17-2.46)		0.59 (0.12-2.80)	
Fourth quartile	49	7 (29.1)	1.31 (0.41-4.19)		1.68 (0.36-7.72)	
3-D value						
First quartile	59	11 (45.8)	1		1	
Second quartile	45	3 (12.5)	0.31 (0.08-1.19)	0.25*	0.10 (0.02-0.72)	0.08
Third quartile	57	5 (20.8)	0.42 (0.14-1.30)		0.40 (0.07-2.31)	
Fourth quartile	43	5 (20.8)	0.57 (0.18-1.79)		0.16 (0.01-2.16)	

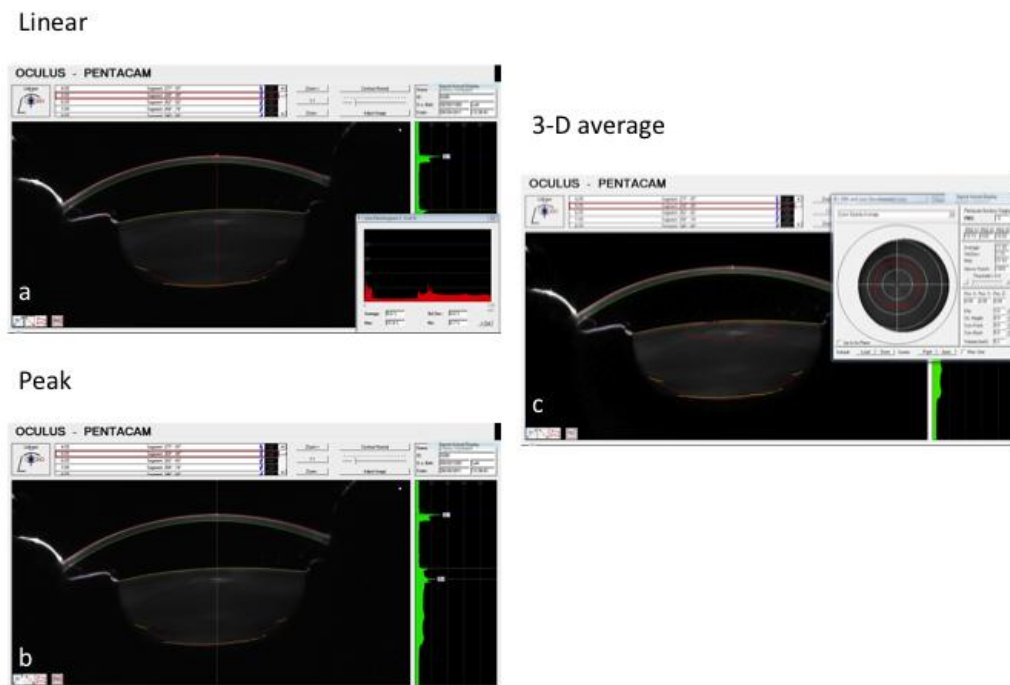
* - Refers to p-value for test of linear trend

d – comparison group are HIV-infected individuals on HAART with a nadir CD4 count \leq 200cells/ μ L

e - Adjusted for age, gender, UV exposure status, smoking status, alcohol intake, income, history of any eye condition, current CD4 count, current and peak viral load, TB status, WHO clinical stage, HAART duration and regimen

FIGURE (FOR MAIN MANUSCRIPT)

Figure 1: Example of Pentacam image acquisition. A) and B) The linear and peak metrics were provided directly from the axis line appearing in the acquired image. The linear metric was the average lens opacity in one plane; the peak reflected the highest lens density within the scan. C) The 3D metric required the observer to select the size and position for analysis, and provided an overall average from the 3-dimensional acquired image.



SUPPLEMENTARY TABLES

Table S1: Comparison of lens density* in HIV-infected and HIV-seronegative groups

Variable	HIV-infected				HIV-seronegative			
	Univariate OR ^a	P-value	Multivariate** OR	P-value	Univariate OR	P-value	Multivariate OR	P-value
Gender								
Male	1		1		1		1	
Female	1.22 (0.60-2.46)	0.58	1.74 (0.58-5.23)	0.32	1.08 (0.54-2.14)	0.83	0.95 (0.30-2.95)	0.92
Age								
Per 10 year increase	14.52 (7.26-20.02)	<0.0001	15.61 (7.34-33.23)	<0.0001	13.14 (6.94-24.85)	<0.0001	16.82 (7.63-37.11)	<0.0001
Income								
<1000 ZAR/month	1		1		1		1	
>1000 ZAR/month	1.56 (0.86-2.86)	0.15	1.28 (0.54-3.06)	0.57	1.02 (0.54-1.90)	0.96	0.35 (0.13-1.04)	0.06
UV exposure								
Outdoor work	1		1		1		1	
Indoor work	0.53 (0.27-1.05)	0.07	0.71 (0.28-1.81)	0.48	0.35 (0.16-0.76)	0.008	0.35 (0.12-1.00)	0.05
Cigarette use								
No	1		1		1		1	
Yes	1.06 (0.46-2.39)	0.90	1.61 (0.49-5.28)	0.43	1.04 (0.55-2.00)	0.90	0.80 (0.26-2.45)	0.69
Alcohol consumption								
Nil	1		1		1		1	
Up to 1L/week	0.91 (0.40-2.08)	0.33	0.75 (0.23-2.40)	0.71	0.89 (0.43-1.85)	0.52	1.40 (0.44-4.43)	0.77
Over 1L/week	1.94 (0.79-4.72)		1.39 (0.39-4.90)		0.64 (0.29-1.40)		1.47 (0.42-5.16)	
History of eye condition								
No	1		1		1		1	
Yes	1.06 (0.48-2.32)	0.89	0.53 (0.16-1.73)	0.29	3.87 (1.61-9.32)	0.002	1.50 (0.43-5.32)	0.53

*Odds ratio for having 'high' lens density – denoted as greater than or equal to the 75th percentile of linear lens density referenced to the control population

a- OR: odds ratio; ** Multivariate OR – adjusted for all other variables in the table.

Table S2: Association of lens density with current CD4 count

Lens density	N	HIV+ on HAART; current CD4 >500 cells/µL (%) ^a	Univariate		Multivariate ^b	
			OR (95%CI)	P-value	OR (95%CI)	P-value
Linear value, all participants						
First quartile (lowest)	50	21 (23.6)	1		1	
Second quartile	57	31 (34.8)	1.65 (0.77-3.54)		1.45 (0.59-3.56)	
Third quartile	53	19 (21.4)	0.77 (0.35-1.71)	0.18	0.71 (0.23-2.14)	0.53
Fourth quartile (highest)	49	18 (20.2)	0.80 (0.36-1.80)		1.03 (0.26-4.01)	
Peak value, all participants						
First quartile	53	23 (25.8)	1		1	
Second quartile	55	26 (29.2)	1.17 (0.55-2.50)		1.24 (0.51-3.00)	
Third quartile	52	22 (24.7)	0.96 (0.44-2.07)	0.43*	1.19 (0.48-2.97)	0.93
Fourth quartile	49	18 (20.2)	0.76 (0.34-1.68)		1.41 (0.50-3.96)	
3-D value, all participants						
First quartile	59	25 (29.1)	1		1	
Second quartile	45	21 (24.4)	1.19 (0.55-2.60)		0.97 (0.37-2.54)	
Third quartile	57	22 (25.6)	0.85 (0.41-1.80)	0.88	0.74 (0.24-2.27)	0.39
Fourth quartile	43	18 (20.9)	0.98 (0.44-2.17)		1.98 (0.44-9.00)	

a- Comparison group are HIV-infected individuals on HAART with a current CD4 count <500cells/µL

b - Adjusted for age, gender, UV exposure status, smoking status, alcohol intake, income, history of any eye condition, nadir CD4 count, current and peak viral load, TB status, WHO clinical stage, HAART duration and regimen * - Refers to p-value for test of linear trend

Table S3: association of lens density with detectable viral load

Lens density	N	HIV+ on HAART; VL>50 copies/ml ^c (%)	Univariate OR (95%CI)	P-value	Multivariate ^d OR (95%CI)	P-value
Linear value, all participants						
First quartile (lowest)	50	8 (24.4)	1		1	
Second quartile	57	7 (21.2)	0.74 (0.25-2.20)		0.89 (0.24-3.31)	
Third quartile	53	10 (30.3)	1.22 (0.44-3.39)	0.82	2.40 (0.51-11.41)	0.19*
Fourth quartile (highest)	49	8 (24.2)	1.02 (0.35-2.99)		3.12 (0.51-19.14)	
Peak value, all participants						
First quartile	53	7 (21.2)	1		1	
Second quartile	55	7 (21.2)	0.96 (0.31-2.95)		0.93 (0.24-3.59)	
Third quartile	52	9 (27.3)	1.38 (0.47-4.02)	0.25*	1.50 (0.41-5.41)	0.19*
Fourth quartile	49	10 (30.3)	1.68 (0.59-4.84)		2.24 (0.58-8.64)	
3-D value, all participants						
First quartile	59	11 (33.3)	1		1	
Second quartile	45	6 (18.2)	0.67 (0.23-1.98)		1.09 (0.29-4.13)	
Third quartile	57	10 (30.3)	0.93 (0.36-2.39)	0.86	1.25 (0.30-5.31)	0.67*
Fourth quartile	43	6 (18.2)	0.71 (0.24-2.09)		1.54 (0.21-11.08)	

c- comparison group are HIV-infected individuals on HAART with viral load <50 copies/ml.

d - Adjusted for age, gender, UV exposure status, smoking status, alcohol intake, income, history of any eye condition, current nadir CD4 count, peak viral load, TB status, WHO clinical stage, HAART duration and regimen

* - Refers to p-value for test of linear trend

SUPPLEMENTARY FIGURES

Figure S1a: Linear metric from lens with minimal opacities

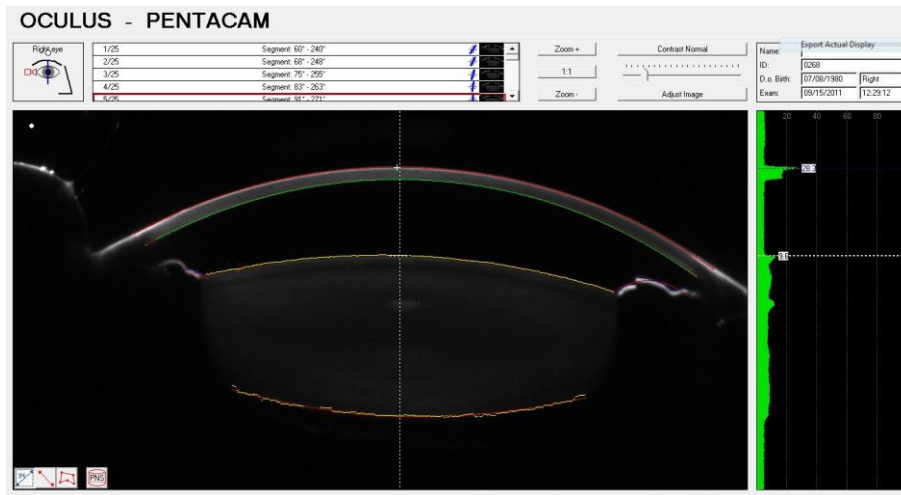


Figure S1b: Peak metric from lens with minimal opacities

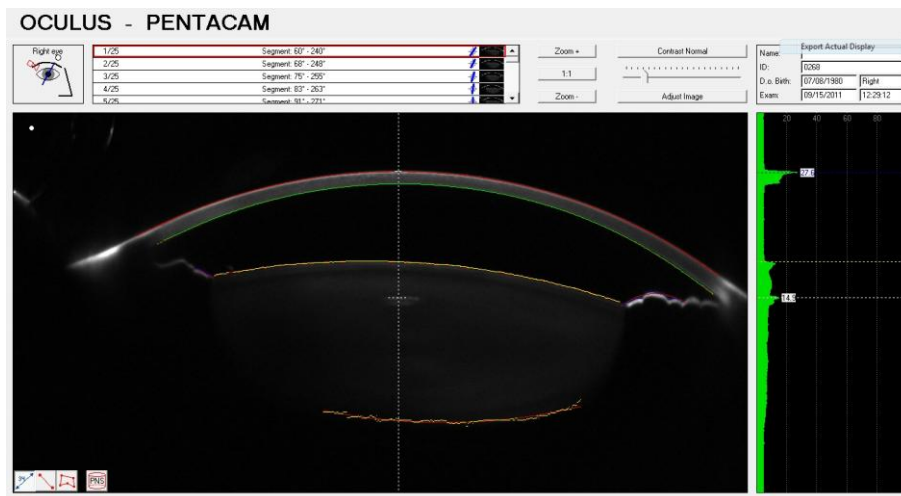


Figure S1c: 3D-average metric from lens with minimal opacities

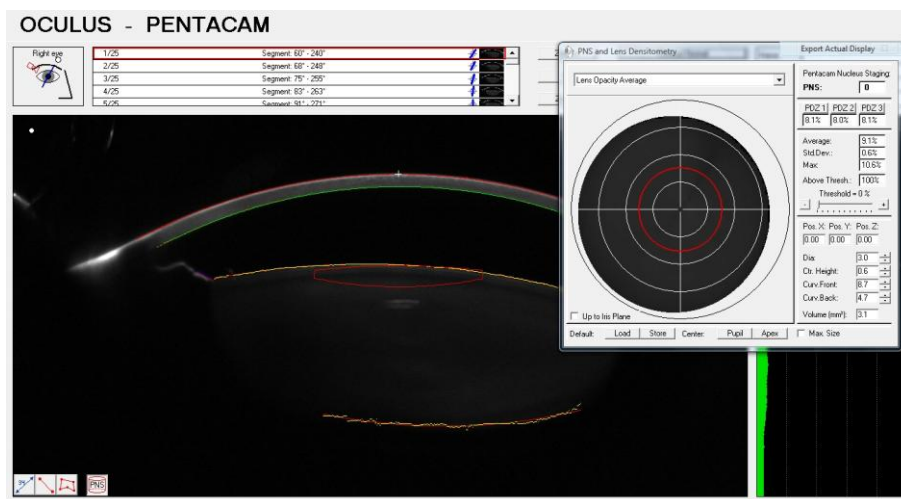


Figure S2a: Linear metric from lens with mid-range lens opacities

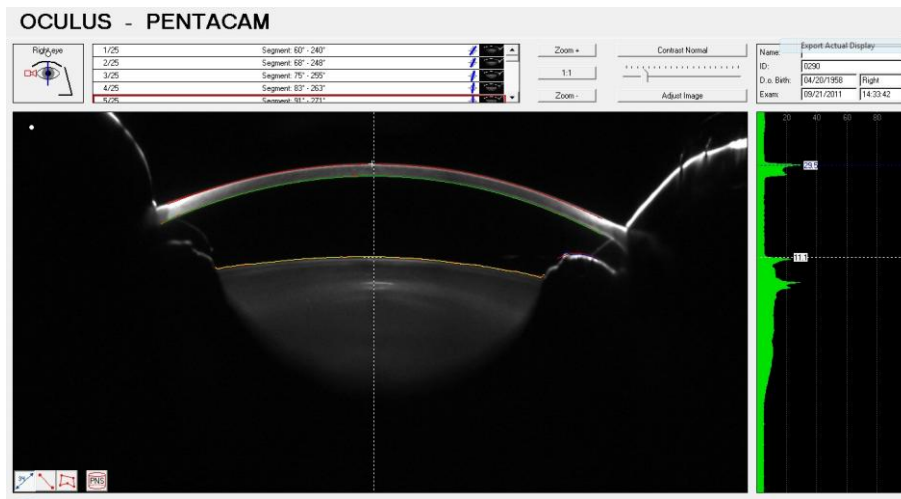


Figure S2b: Peak metric from lens with mid-range lens opacities

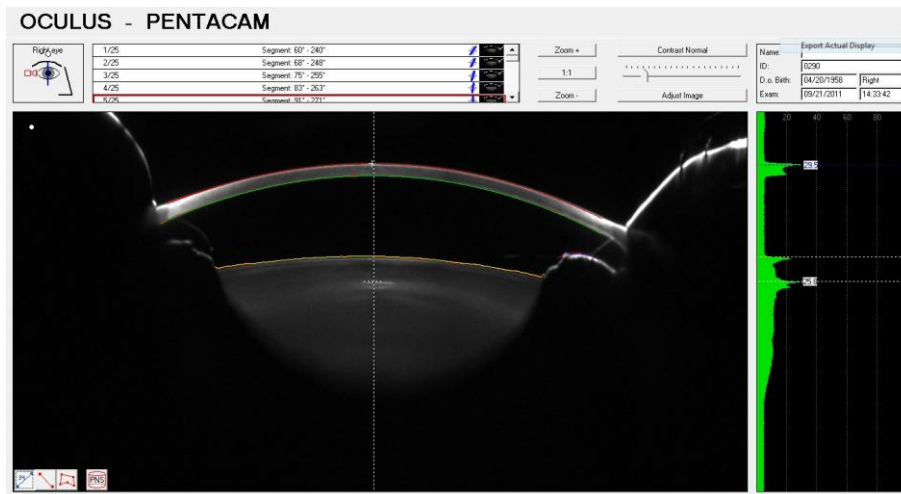


Figure S2c: 3D-average metric from lens with mid-range lens opacities

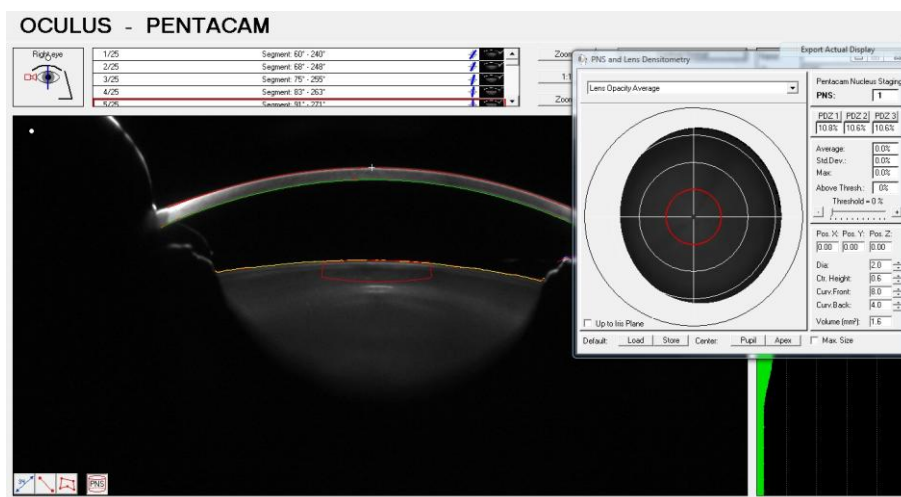


Figure S3a: Linear metric from lens with high lens opacities

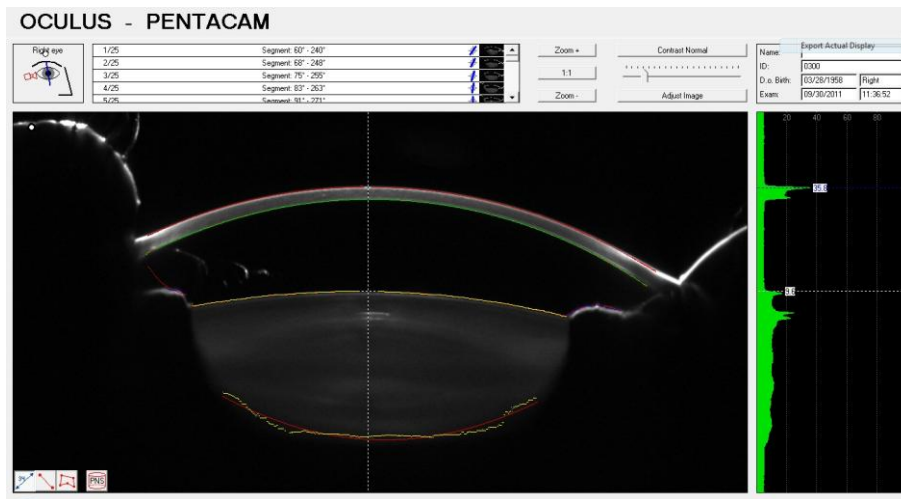


Figure S3b: Peak metric from lens with high lens opacities

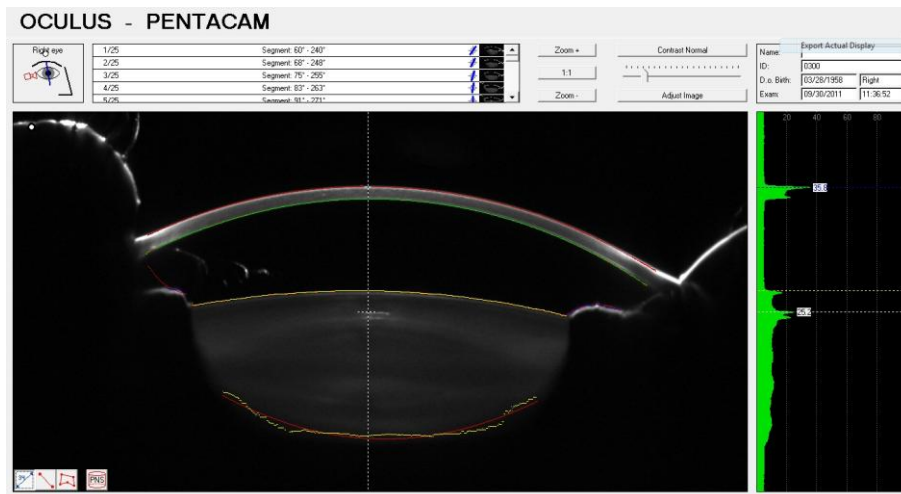


Figure S3c: 3D-average metric from lens with high lens opacities

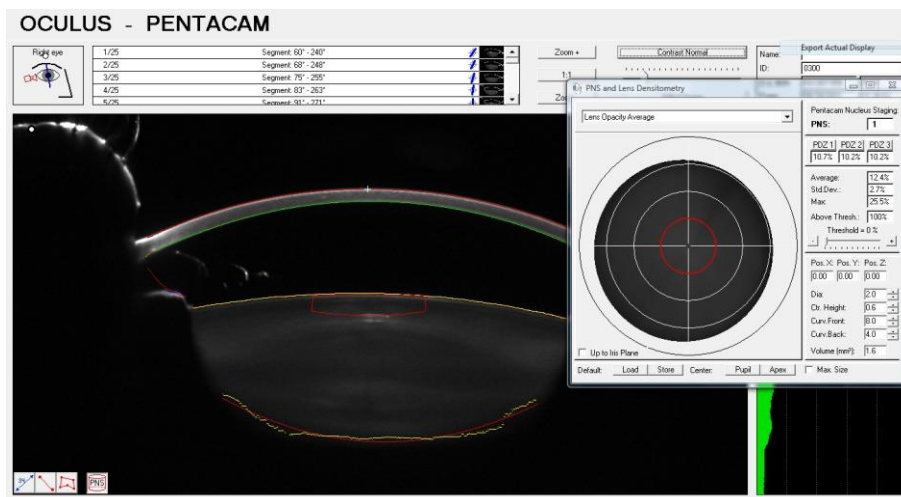


Figure S4: Example of very high lens density (clinical cataract)

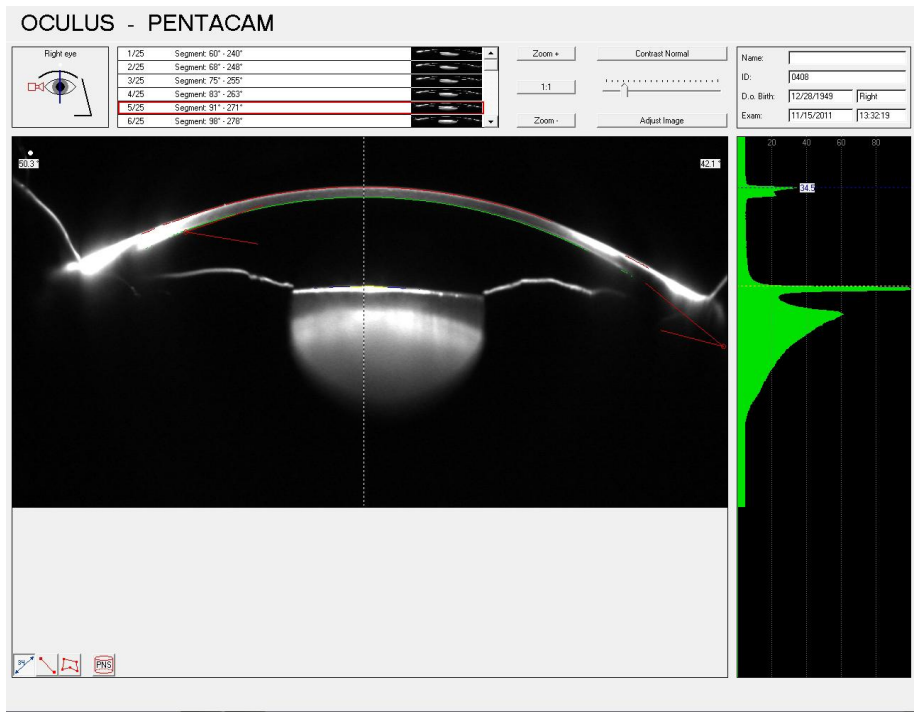
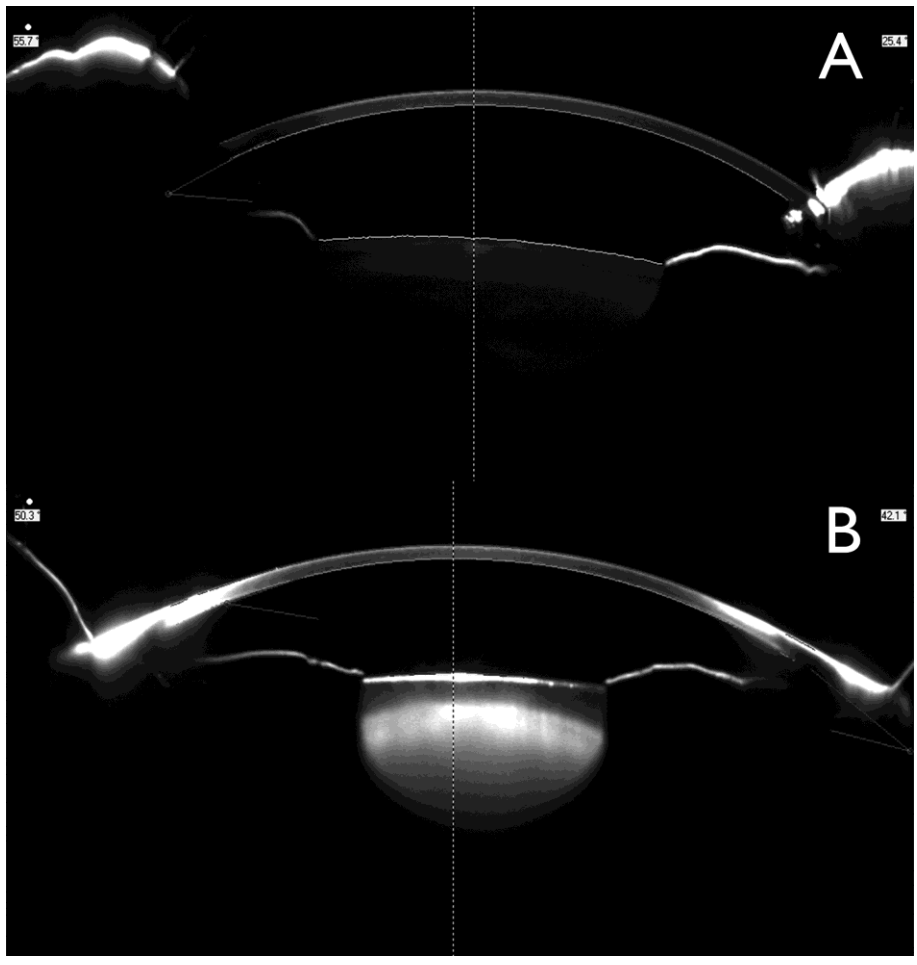
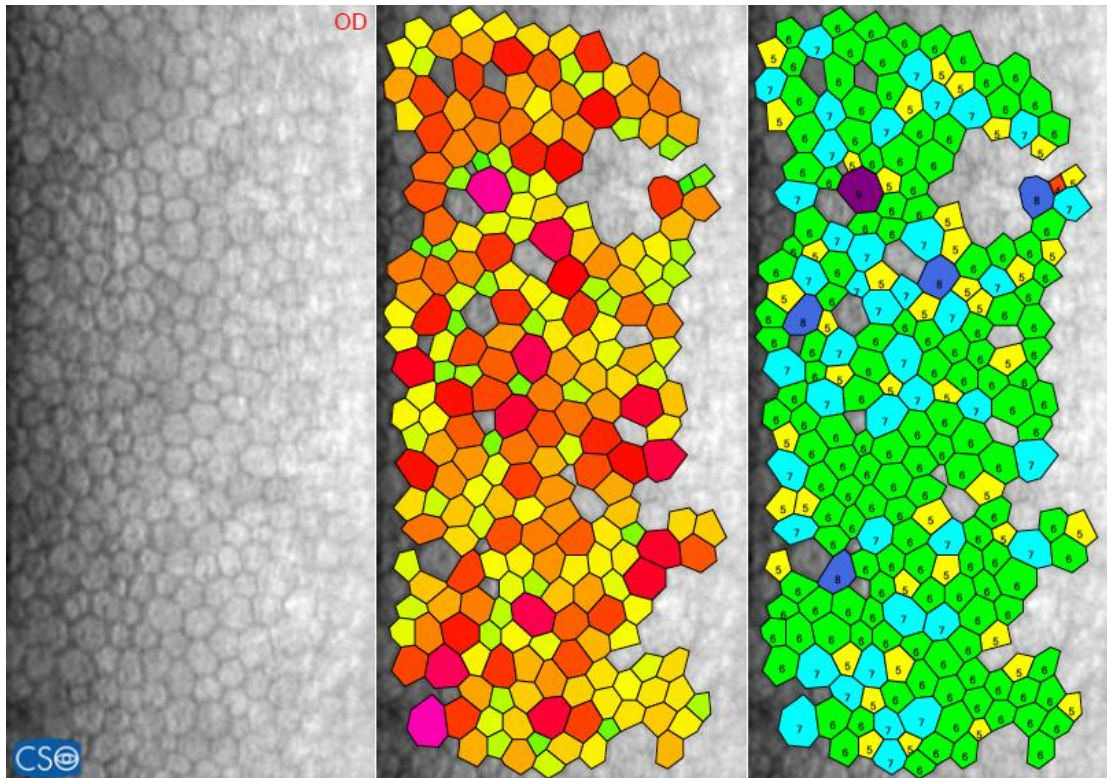


Figure S5: Comparison of lens with minimal (a) and high (b) lens opacities



Chapter 9

Corneal endothelial cells provide evidence of accelerated cellular senescence associated with HIV infection: a case-control study



Corneal endothelial cells captured by specular microscopy with polymegathism and pleomorphism indices

Research paper evaluating corneal endothelial cell parameters known to be associated with biological aging, and cellular senescence markers in HIV-infected adults.

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **PLOS ONE**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, SD Lawn, PG Shiels, HA Weiss, C Cook, R Wood, CE Gilbert
 - 2.3. Stage of publication – ~~Not yet submitted/~~**Submitted/**~~Undergoing revision from peer reviewers' comments/~~In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments and conducted the data collection. I performed all specular microscopy imaging and clinical examination. I performed the statistical analyses with advice from Helen Weiss. Paul Shiels' laboratory team were responsible for measurement of CDKN2A levels. Monica Vogt measured the 8OHdG levels. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



TITLE PAGE

Corneal endothelial cells provide evidence of accelerated cellular senescence associated with HIV infection: a case-control study

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Abstract

Background

Cellular senescence may be a key factor in HIV-related premature biological aging. We assessed features of the corneal endothelium that are known to be associated with biological aging, and cellular senescence markers in HIV-infected adults.

Methods:

Case-control study of 242 HIV-infected adults and 249 matched controls. Using specular microscopy, the corneal endothelium was assessed for features of aging (low endothelial cell density [ECD], high variation in cell size, and low hexagonality index). Data were analysed by multivariable regression. CDKN2A (a cell senescence mediator) and 8-hydroxy-2'-deoxyguanosine (an oxidative DNA damage marker) were measured in peripheral blood leukocytes.

Results:

The median age of both groups was 40 years. Among HIV-infected adults, 88% were receiving antiretroviral therapy (ART); their median CD4 count was 468 cells/ μ L. HIV infection was associated with increased odds of variation in cell size (OR=1.67; 95% CI: 1.00-2.78, $p=0.04$). Among HIV-infected participants, low ECD was independently associated with current CD4 count <200 cells/ μ L (OR=2.77; 95% CI: 1.12-6.81, $p=0.03$). In participants on ART with undetectable viral load, CDKN2A expression and 8-OHDG levels were higher in those with accelerated aging, as reflected by lower ECD.

Conclusions:

The corneal endothelium shows features consistent with HIV-related accelerated senescence, especially among those with poor immune recovery.

Abstract word count: 200

Keywords: corneal endothelium; HIV; aging; immunosenescence; senescence
polymegathism;

Suggested running head

Senescent corneal endothelial cells in HIV

MAIN TEXT

Introduction

Patients receiving antiretroviral therapy (ART) are at increased risk of age-related non-AIDS morbidity and mortality compared with HIV-seronegative persons [1,2]. It is speculated that as those with HIV age chronologically, they are also likely to undergo accelerated biological aging, mediated by increased cellular senescence. This may be due to replicative senescence (a state of irreversible cellular growth arrest) and stress-induced premature senescence (SIPS) from exposure to environmental stresses, including oxidative stress [3]. Senescent cells adopt an enlarged morphology and secrete inflammatory factors leading to low-level, chronic inflammation described as the senescence-associated secretory phenotype (SASP) [4].

The eye may be a useful site for investigating cellular dynamics of aging, as the corneal endothelium can be readily visualized using non-invasive techniques. The corneal endothelium is a monolayer of mosaic-like cells which lines the inner surface of the cornea. Human corneal endothelial cells (HCEC) do not have substantial replicative potential *in vivo* and their form and quantity influence the health of the cornea [5]. Cells vary from 4 to 6 μm in thickness, are up to 20 μm in width, and have a stable, metabolically efficient hexagonal shape [6]. A key function of the corneal endothelium is to maintain corneal transparency via an ionic 'pump'. Loss of HCEC beyond a critical threshold results in corneal oedema and loss of visual acuity.

HCEC can be viewed non-invasively via specular microscopy which uses specular reflection [7]. Three endothelium parameters are commonly assessed. Firstly, the number of cells within a defined area - endothelial cell density (ECD), which

decreases [8-10] throughout life at an average rate of 0.3-0.6%/year [5,11]. Mean cell density is approximately 3400 cells/mm² at age 15 years declining to approximately 2300 cells/mm² by 85 years [12]. The endothelial response to gradual cell loss is spreading and migration of neighbouring cells, leading to an increase in overall cell size and loss of hexagonal shape [5]. Thus, the second parameter that can be measured is variation in cell size (*polymegathism*) which is- measured using the coefficient of variation (CV) of cell area, which is the ratio between the observed standard deviation and the arithmetic mean of all the cells examined. An average value is less than 35. Variation in cell size increases with increasing chronological age. The final parameter is the hexagonality index – the proportion of cells with 6 sides and this decreases with increasing chronological age [13].

Reduction in proliferative capacity of HCEC is partly mediated by an age-related increase in expression of the cell cycle mediator CDKN2A [14,15] that functions to hold a cell in a state of growth arrest. Increasing levels of CDKN2A transcriptional expression occur with increasing age, in solid organs and peripheral blood leukocytes [16-19]. Reduction in HCEC proliferative capacity may also result from nuclear oxidative damage, which can be assessed by 8-hydroxy-2'-deoxyguanosine (8-OHDG) levels, a physical marker of oxidative DNA damage [3,20]. If premature cellular senescence occurs in HIV infection, senescent changes in HCEC may be evident in HIV-infected individuals compared to age-matched uninfected controls. Nadir CD4 count, current CD4 count and ART duration have frequently been reported as predictors of increased risk of systemic age-related morbidities in HIV-infected people [21-24]. We have previously reported from a South African population that HIV infection is associated with a functional phenotype

consistent with frailty [25], and that changes in retinal vessel calibre (as a proxy for systemic vasculature) are consistent with accelerated aging and increased cardiovascular risk [26]. We now report on the cellular aspects of aging within this study cohort, using corneal endothelial cells as readily accessible model of cellular senescence. The objective of this study was to assess differences and identify predictors of HCEC parameters and markers of cellular senescence in a cohort of HIV-infected individuals in comparison to healthy controls.

Methods

Study participants

HIV-infected participants aged 30 years and above were enrolled from a community-based HIV treatment centre in Nyanga district of Cape Town, South Africa [27,28]. Participant recruitment has been reported in detail elsewhere [25]. In brief, all participants had a confirmed serological diagnosis of HIV and were either about to commence ART (ART-naïve), or were already on first-line ART. A control group of HIV-seronegative participants was recruited using frequency-matching by gender and 5-year age categories.

The study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee and the University of Cape Town Faculty of Health Sciences Ethics Committee, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants

Data collection

Socio-demographic information and medical history were obtained by questioning participants in their first language (Xhosa or English). Data were also collected on

exposures that are known to affect aging and age-related parameters (e.g. smoking, work location as a proxy for UV exposure). Clinical information was obtained from medical case notes where required. All participants underwent a full ophthalmic examination including measurement of visual acuity, evaluation by slit lamp microscopy (for anterior segment structures e.g. cornea, anterior chamber) and indirect ophthalmoscopy (for retina and vitreous).

Endothelium assessment

A non-contact specular microscope was used (SP02, CSO; Florence, Italy) in automatic release mode to reduce operator-dependent variables. The operator focused and aligned a real-time image of the participant's eye. The instrument captured the endothelium in the central corneal area, and was calibrated to account for varying thickness of the central cornea (which has the potential to affect endothelial cell counts). The instrument software automatically calculated the quality and reliability of a captured image; if an image was of poor quality (i.e. not 'ok' on the image quality specification), the measurement was repeated. Participants who had corneal pathology or evidence of past or current intraocular inflammation were excluded from analysis. Endothelial parameter values were extracted from the image captures in a masked fashion. Two assessments were taken per eye and the mean was used in analyses. Figure 1 shows an example of the output.

CDKN2A expression determination and 8OHdG measurement

Relative quantitative real-time PCR (qRT-PCR) was used to estimate mRNA levels corresponding to the candidate senescence associated gene - CDKN2A. Expression levels were measured against a reference HPRT housekeeping gene on an ABI

Prism(R) 7500 Sequence Detection System. Sequences of human TaqMan™ Primer/Probe sets were designed by Primer Express algorithm (Applied Biosystems, Austin, TX, USA). The comparative threshold cycle method ($\Delta\Delta CT$) was employed to quantify relative gene expression. The quantification result was transformed to an exponential value, $2^{-\Delta\Delta Ct}$ [29] where Ct is the threshold cycle, or the cycle when the product was first detected. Prior to this quantitative study we demonstrated that the efficiency of amplification of reference (HPRT) and test genes were approximately equal (data not shown). Plasma 8-OHDG concentration was quantified using ELISA kits (OxiSelect, Cell Biolabs, CA, USA) according to manufacturer's instructions.

Statistical analyses

One eye per person was randomly selected for analyses. Where an eye was not available, for example due to trauma, the contralateral eye was used. Endothelium parameters were assessed as categorical variables by division into quartiles defined in the HIV-seronegative participants. The association of HIV status with these quartiles was assessed in univariable and multivariable logistic regression models adjusted for risk factors and *a priori* confounders (UV exposure, smoking). Linear regression analyses were also performed to assess the rate of endothelial cell loss per year in the two groups using age as a continuous variable. The endothelial parameter phenotypes were further defined with binary categorization to denote 'aging' as follows: low endothelial cell count (<25th percentile: <2461 cells/mm²), high polymegathism (>75th percentile: >39), and low hexagonality index (<25th percentile: <46). Serum biomarkers (CDKN2A and 8-OHDG) were analysed as continuous variables and multiple linear regression models were used to examine the relationships of biomarker

expression with endothelial cell parameters. All analyses were performed with Stata 11 IC (Stata Corp, USA).

Results

Participant characteristics

Characteristics of the 242 HIV-infected individuals and 249 uninfected age/gender frequency matched HIV-seronegative individuals are reported in Table 1. HIV-infected participants tended to have a greater income (attributable to a government health-related grant), to be non-smokers, and reported less consumption of alcohol than HIV-seronegative individuals. HIV-infected participants were almost twice as likely to have a history of an eye condition ($p=0.01$), but there was little difference in visual acuity (Table 1). None of the participants had undergone intraocular surgery in either eye or had evidence of clinically active uveitis, both of which could have affected the endothelium. Among the HIV-infected participants, 88% were receiving ART, the current median CD4 count was 468 cells/ μL (interquartile range: [IQR]: 325-607) and 84% had an undetectable plasma viral load (VL –defined as <50 copies/mL).

Corneal endothelium assessment

Three participants had corneal pathology precluding accurate image acquisition (inactive corneal infiltrates of both eyes), leaving 488 eyes for analysis. Endothelium parameter images of sufficient quality were available for 468 (95.9%) eyes.

Acquisition of sub-optimal images was related to participant movement and rapid blinking during image capture. Poor quality images were more frequent in

participants with a history of an eye condition compared to those without (9.4% vs. 3.3%, $p=0.02$) and in those who had HIV infection (5.8% vs. 2.4%, $p=0.06$).

Endothelial cell density

Median cell density values were 2660 cells/mm² (IQR: 2433-2851) in HIV-infected participants and 2614 cells/mm² (IQR: 2460-2802) in HIV-seronegative individuals ($p=0.56$). The age-adjusted rate of endothelial cell loss was greater in HIV-infected participants compared to HIV-seronegative individuals (0.30% per year vs. 0.15%), although this did not reach statistical significance (p -interaction=0.16). Table 2 reports the association of the endothelial cell parameters with HIV status. Table 3 reports associations of socio-behavioural and clinical factors with low cell density within the study population. Increasing age was strongly associated with low cell density, both in univariable and multivariable analysis (p -trend=0.001). In addition to increasing age and male gender, a key finding was that low current CD4 counts were independently associated with low cell density among HIV-infected individuals (Table 4). Participants with CD4 counts <200 cells/ μ L were almost three times more likely to have a low cell density (OR=2.77; 95%CI: 1.12-6.81). This association was strengthened when restricted to participants on ART ($n=198$) (OR=4.63; 95%CI: 1.48-14.50– data not shown).

Endothelial cell polymegathism (variation in cell size)

The median variation in cell size (polymegathism) was 36 (IQR: 33-39) in HIV-infected participants and 35 (IQR: 32-38), $p=0.07$ in HIV-seronegative individuals. HIV was associated with higher polymegathism quartiles in univariable analyses (p -trend=0.04), and this association strengthened after adjustment (p -trend=0.006)

(Table 2). HIV infection was also associated with high polymegathism as a binary variable (OR=1.67, 95%CI 1.00-2.78) (Table 5). Increasing age, an outdoor work location and increased duration of smoking were also associated with high polymegathism. However, despite the association of HIV infection with this variable, HIV-related covariates were not related. Independent predictors in HIV-infected individuals were outdoor work (OR=2.20; 95%CI: 1.00-4.90) and cigarette smoking (OR=3.41; 95%CI: 1.13-10.31) (Table 4).

Hexagonality index (cell shape)

The median hexagonality index was similar in both groups (50 [IQR: 45-54] vs. 50 [IQR: 46-54], $p=0.55$). Low hexagonality index was associated with increasing age (p -trend=0.006) and outdoor work location (OR=1.70; 95%CI 1.04-2.78) but there was little evidence of an association with HIV infection (OR=1.40, 95%CI 0.90-2.17). In HIV-infected individuals, low hexagonality index was associated with outdoor work (OR=1.97; 95%CI: 1.00-3.95); and inversely related with a history of an eye condition (OR=0.18; 95%CI: 0.07-0.62) (Table 4). No HIV-related covariates were associated with this endothelial parameter.

Association between markers of cellular senescence and endothelial cell density

CDKN2A measurements were available for 430 participants, (221 HIV+/209 HIV-). CDKN2A transcriptional expression was higher in HIV-infected participants compared to HIV-seronegative individuals (0.46 vs. 0.37, $p=0.007$). In ART-treated participants with suppressed viral load (<50 copies/ml; $n=155$) adjusted CDKN2A expression was higher in those with low cell density compared to those with high cell density (0.57 vs. 0.43, $p=0.04$). There was no evidence to suggest an association

between CDKN2A expression and cell density in HIV-seronegative individuals (p=0.33).

8-hydroxy-2'-deoxyguanosine (8-OHDG) levels were available for 70 individuals. 8OHDG levels were positively associated with CDKN2A expression in HIV-seronegative individuals (r=0.13, p=0.33). Mean levels were 0.21ng/ml in HIV-seronegative individuals and 0.22ng/ml in HIV-infected individuals (p=0.83). In participants on ART with undetectable viral load (n=28), those with low ECD count had higher 8OHDG levels compared to those with high cell density (0.25ng/ml vs. 0.19ng/ml, p=0.04). There was no association between 8OHDG levels and cell density in HIV-seronegative individuals.

Discussion

We have already demonstrated in this South African study population that HIV infection is associated with an increased risk of frailty, providing evidence that this functional phenotype is associated with HIV-related accelerated aging [25]. We have also investigated for evidence of aging at an organ/systems level using the retinal vessels as a model of the systemic vasculature, and our findings suggested that HIV infection is associated with changes in the retinal vasculature that may reflect premature aging and increased cardiovascular risk [26]. These previous studies both provided important evidence that HIV infection is associated with accelerated senescence. The present study further builds on these findings, using corneal endothelial cells to represent a readily accessible cellular model of senescence. Here we have demonstrated that HIV-infected individuals have an increased risk of greater

corneal endothelial cell size variation (polymegathism) when compared with HIV-seronegative individuals. Furthermore, HIV-infected individuals with a current CD4 count <200 cells/ μ L were more likely to have low endothelial cell densities than those with higher CD4 counts. These data were corroborated by markers of cellular damage and senescence which were found to be higher in virally suppressed ART-treated individuals with low cell densities. These findings provide further evidence of accelerated aging in HIV, and that this may be influenced by the level of immunodeficiency, as reflected by CD4 count.

Independent predictors of low cell density in HIV-infected individuals included age (as anticipated), male gender and low current CD4 count. The relationship with male gender is likely to be related to the anti-oxidant and anti-inflammatory properties of oestrogen [20], while the relationship with low current CD4 count suggests that poor immune restoration may accelerate senescence. Our finding that UV exposure and smoking were also independent predictors of polymegathism in the overall study population and in HIV-infected individuals supports the findings of others that stress, including oxidative stress, is likely to be an important contributor to cellular senescence mechanisms in HCECs [30]. Our observation of increased CDKN2A levels in PBLs from HIV-infected individuals compared with HIV-seronegative individuals supports the mechanism of systemic oxidative stress. Furthermore, exposure to cigarette smoke has been demonstrated to degrade the function of the cornea through generation of reactive oxygen species [31]. It is surprising that polymegathism was not associated with current CD4 count or other HIV-related covariates in our study. This may suggest that 'stress' induced by HIV-related chronic inflammation may lead to HCEC senescence, the initial features

of which are an enlarged (or variation in) morphology. Severe immunodeficiency (manifest as a low CD4 count) may be associated with later features of the senescence pathway, ultimately leading to cell cycle arrest and cell loss.

Despite the association of greater polymegathism (variation in cell size) with HIV, we did not detect a difference in cell density between the two groups. The endothelial response to gradual cell loss is spreading and migration of neighbouring cells with an increase in overall cell size and adoption of a non-hexagonal shape [5]. Therefore an overall decrease in ECD might have been expected. However, as increased polymegathism reflects a *variation* in cell size (not simply an increase in cell size), similar ECD between the two groups is not an unusual finding: ECD can remain the same with different levels of polymegathism. Other studies have reported similar findings [32] and patients with diabetes develop increased polymegathism while retaining normal endothelial cell density for their age [33]. Stress to the endothelium can also lead to cell border changes, with cells expressing a large anterior surface area with a small posterior surface area or vice versa [34]. Capturing a two-dimensional image with the specular microscope may, therefore, provide unchanged cell density measures despite greater polymegathism.

An aggregate of phenotypes has been described for senescent cells [4,35], including irreversible growth arrest, an enlarged morphology, expression of CDKN2A and the senescence-associated secretory phenotype (SASP). This cell phenotype is associated with secretion of cytokines, growth factors and proteases that can lead to low-level chronic inflammation that is a feature of normal aging, and also a key feature in HIV infection [36]. In our study, higher CDKN2A expression and 8OHdG

levels in peripheral blood leukocytes were observed in participants on ART with undetectable viral load and low endothelial cell density, suggesting that accelerated cellular senescence may be occurring systemically. Markers of cellular senescence have also been detected within endothelial corneal tissue, from human subjects and mouse senescence models, showing greater CDKN2A expression with increasing age [37,38]. In addition, HCECs exhibit signs of oxidative DNA damage, and significantly higher levels of 8OHdG have been reported in corneas from older donors compared with younger donors [3]. Our findings suggest that corneal endothelial cells in HIV-infected individuals exhibit some of the phenotypic features of senescent cells. Consequently, they would be expected to express more CDKN2A and have the associated SASP compared to HIV-seronegative individuals.

A key strength of our study is the inclusion of an age- and gender-matched control group with a similar socio-demographic profile to the HIV-infected participants. The hypothesis of accelerated aging in HIV has received criticism due to limitations in characterization of participants, in particular the possibility of differential exposure to potential risk factors between HIV-infected and uninfected populations [24,39,40]. By recruiting from the same community, we reduced the likelihood of differential risk exposure. HCEC change morphology and assume an 'aged phenotype' in several chronic systemic diseases (e.g. renal failure and diabetes) [41] [42,43], suggesting that they may be useful in assessing cellular senescence, particularly as measurement is objective and non-invasive.

There are some limitations to our study. Although our hypothesis relates to accelerated aging in HIV, it is possible that subclinical intraocular inflammation could

account for the observed relationship between lower CD4 counts and low endothelial cell density. HIV can replicate within the eye causing uveitis [44], and a low CD4 count is a risk factor for immune recovery uveitis (IRU) [45] which could arise in patients with a history of cytomegalovirus (CMV) infection and could potentially cause subclinical endothelial damage. However, we did not detect any evidence of IRU, and we have previously shown the prevalence of CMV retinitis to be very low within this population [46]. Therefore, our data support the hypothesis of premature aging in line with systemic co-morbidities [21,47]. Another limitation is that misclassification of smoking and alcohol consumption status may have occurred, with HIV-infected participants wanting to demonstrate 'healthy behaviour', which could have led to confounding. Our measure of location of work as a proxy measure of ultra-violet exposure may also have been confounded by socio-economic status. Finally, CDKN2A expression and 8OHdG levels were determined in peripheral blood leukocytes, which may not directly reflect levels in corneal tissue. However, a number of studies indicate that CDKN2A transcriptional expression increases with increasing chronological age in both leukocytes and solid organs [16,17,19].

In summary, corneal endothelial cells demonstrate features of senescence in HIV-infected individuals, suggesting HIV infection contributes towards accelerated cellular senescence. Evaluation of corneal endothelial cells in longitudinal studies of HIV-related accelerated biological aging may reveal further insights into the mechanisms of cellular senescence in this population.

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Author contributions:

SP: conceived and designed the experiments; wrote first draft of manuscript; statistical analysis; critical and intellectual revision of further drafts

SDL: critical and intellectual revision of further drafts

PGS: designed the experiments; laboratory analyses; critical and intellectual revision of further drafts

CC: critical and intellectual revision of further drafts

HW: assistance with statistical analysis: critical and intellectual revision of further drafts

RW: critical and intellectual revision of further drafts

CG: designed the experiments; critical and intellectual revision of further drafts

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Table 1: Characteristics of study participants

Variable	HIV-infected participants (242) % (n)	HIV-seronegative individuals (249)% (n)	P-value
Age (mean±SE)	41.2±0.5	42.5±0.6	0.10
Age, years by group			
	30-39	119 (49.2)	117 (47.0)
	40-49	81 (33.5)	79 (31.7)
	>50	42 (17.4)	53 (21.3)
Male gender	25.6 (62)	24.1 (60)	0.70
Working location			
	Indoors	35.1 (85)	28.1 (70)
Income			
	>ZAR1000/month	42.2 (102)	31.7 (79)
Smoking status			
	Non-smoker	85.5 (207)	72.7 (181)
	<10 years	4.6 (11)	12.9 (32)
	>10 years	9.9 (43)	14.5 (36)
Alcohol			
	Yes	30.2 (73)	44.2 (110)
History of eye condition			
	Yes	16.9 (41)	9.3 (23)
Visual acuity (presenting)	20/25	20/20	0.68
% with visual acuity <20/40	6.2 (15)	10.0 (25)	0.12
HIV-related characteristics (n=242)			
WHO stage			
	1/2	27.3 (66)	
	3/4	72.7 (176)	
ART* naïve	12.0 (29)		
CD4 count in ART naïve group (n=29)**	170 (84-201)		
Log ₁₀ VL in ART naïve group (n=19)***	4.81 (4.11-5.14)		
Current CD4 count in ART group (n=213)**	468 (325-607)		
Nadir CD4 count in ART group **	127 (76-171)		
% with detectable VL in ART group	16.0 (34)		
Peak Log ₁₀ VL in ART group ***	4.56 (3.85-4.98)		
Duration of ART, months	58 (34-75)		
ART Regimen			
	Containing AZT/3TC	59.6 (127)	
	Other	40.3 (86)	

*ART= Anti-retroviral therapy; **=cells/μl; ***=copies/ml

Table 2: Association of endothelial cell parameters with HIV status

*=p-value: test for trend; ** Adjusted for age, gender, UV exposure, BMI, alcohol and cigarette smoking status, any history of an eye condition

Endothelial cell parameters	N (468)	HIV-infected participants (%)	Univariate		Multivariate**	
			OR (95%CI)	P-value	OR (95%CI)	P-value
Endothelial cell density (ECD)						
First quartile (lowest)	125	64(28.3)	1		1	
Second quartile	98	38 (16.8)	0.60 (0.35-1.03)	0.18	0.62 (0.35-1.10)	0.22
Third quartile	119	58 (25.7)	0.91 (0.55-1.50)		0.93 (0.55-1.57)	
Fourth quartile (highest)	126	66 (29.2)	1.05 (0.64-1.72)		1.10 (0.65-1.85)	
Polymegathism (variation in cell size)						
First quartile	121	51 (22.5)	1		1	
Second quartile	129	59 (26.1)	1.16 (0.70-1.91)		0.99 (0.58-1.68)	
Third quartile	103	54 (23.9)	1.51 (0.89-2.57)	0.04*	1.52 (0.87-2.67)	0.006*
Fourth quartile	115	62 (27.4)	1.61 (0.96-2.69)		1.99 (1.15-3.45)	
Hexagonality index (cell shape)						
First quartile	142	74 (32.7)	1		1	
Second quartile	117	56 (24.8)	0.84 (0.52-1.36)		0.76 (0.46-1.27)	
Third quartile	109	47 (20.8)	0.70 (0.42-1.15)	0.57	0.59 (0.34-1.00)	0.27
Fourth quartile	100	49 (21.7)	0.88 (0.53-1.47)		0.81 (0.48-1.39)	

Table 3: Association of low endothelial cell density in study population (n=468)

*=test for trend; **- Adjusted for all other variables in model

Variable	Univariate		Multivariate**	
	OR (95%CI)	P-value	OR (95%CI)	P-value
HIV infection				
	No	1	1	
	Yes	1.17 (0.78-1.77)	1.14 (0.74-1.76)	0.56
Gender				
	Male	1	1	
	Female	0.74 (0.47-1.18)	0.66 (0.39-1.14)	0.14
Age (years)				
	30-39	1	1	
	40-49	1.60 (0.99-2.57)	1.60 (0.98-2.61)	
	>50	2.41 (1.41-4.15)	2.43 (1.39-4.24)	0.001*
Work location				
	Outdoor worker	0.96 (0.62-1.49)	0.87 (0.55-1.37)	
	Indoor worker	1	1	0.55
Cigarette smoker				
	No	1	1	
	Yes	0.87 (0.52-1.46)	0.66 (0.34-1.24)	0.20
Alcohol consumption				
	No	1	1	
	Yes	0.89 (0.58-1.36)	0.83 (0.51-1.36)	0.46
History of eye condition				
	No	1	1	
	Yes	1.71 (0.96-3.06)	1.41 (0.77-2.57)	0.27

Table 4: Multivariable models to demonstrate association of endothelial cell parameters in HIV-infected individuals (n=217)

§ - Adjusted for all other variables in model; *=test for trend

Variable	Low endothelial cell density [§]		High polymegathism [§] (cell variation)		Low hexagonality index [§] (cell shape not 6-sided)	
	OR	P-value	OR	P-value	OR	P-value
Gender						
Male	1		1		1	
Female	0.43 (0.19-0.91)	0.04	2.19 (0.81-5.86)	0.12	0.99 (0.44-2.29)	0.98
Age (years)						
30-39	1		1		1	
40-49	2.84 (1.35-5.97)		1.00 (0.43-2.31)		0.63 (0.30-1.34)	
>50	5.42 (2.21-13.26)	<0.0001*	2.44 (0.97-6.13)	0.09*	1.69 (0.71-3.98)	0.12
Work location						
Outdoor worker	0.57 (0.29-1.12)		2.20 (1.00-4.90)		1.97 (1.00-3.95)	
Indoor worker	1	0.11	1	0.05	1	0.05
Cigarette smoker						
No	1		1		1	
Yes	1.04 (0.39-2.78)	0.93	3.41 (1.13-10.31)	0.03	1.09 (0.39-3.06)	0.87
History of eye condition						
No	1		1		1	
Yes	1.04 (0.44-2.49)	0.93	0.95 (0.37-2.49)	0.93	0.18 (0.05-0.62)	0.01
WHO clinical stage						
1/2	1		1		1	
3/4	1.58 (0.71-3.51)	0.26	1.88 (0.80-4.54)	0.16	1.11 (0.52-2.33)	0.79
Peak viral load						
<10,000 copies	1		1		1	
>10,000 copies	0.65 (0.32-1.31)	0.23	0.87 (0.40-1.86)	0.71	0.84 (0.42-1.69)	0.62
Current CD4 count						
<200 cells/ μ L	2.77 (1.12-6.81)		1.30 (0.49-3.48)		0.62 (0.24-1.61)	
>200 cells/ μ L	1	0.03	1	0.60	1	0.33

Table 5: Association of high polymegathism (increased variation of cell size) in study population (n=468)

*=test for trend; § - Adjusted for all other variables in model

Variable	Univariate		Multivariate§	
	OR (95%CI)	P-value	OR (95%CI)	P-value
HIV infection				
No	1		1	
Yes	1.26 (0.78-2.01)	0.34	1.67 (1.00-2.78)	0.04
Gender				
Male	1		1	
Female	1.54 (0.86-2.78)	0.15	2.79 (1.35-5.77)	0.006
Age (years)				
30-39	1		1	
40-49	1.43 (0.83-2.48)		1.31 (0.74-2.33)	
>50	2.33 (1.28-4.25)	0.02	2.33 (1.23-4.40)	0.01*
Work location				
Outdoor worker	1.78 (1.02-3.10)		1.76 (.100-3.12)	
Indoor worker	1	0.04	1	0.05
Cigarette smoker				
Nil	1		1	
5yrs or less	1.91 (0.72-5.07)		2.07 (0.71-6.02)	
6-15	1.27 (0.46-3.16)		2.34 (0.73-7.44)	
16-20	1.69 (0.65-4.44)		2.47 (0.83-7.39)	
>20	1.87 (0.75-4.64)	0.43	2.72 (0.9108.12)	0.02*
Alcohol consumption				
No	1		1	
Yes	0.77 (0.47-1.22)	0.26	0.70 (0.39-1.24)	0.22
History of eye condition				
No	1		1	
Yes	1.71 (0.96-3.06)	0.07	0.80 (0.38-1.72)	0.57

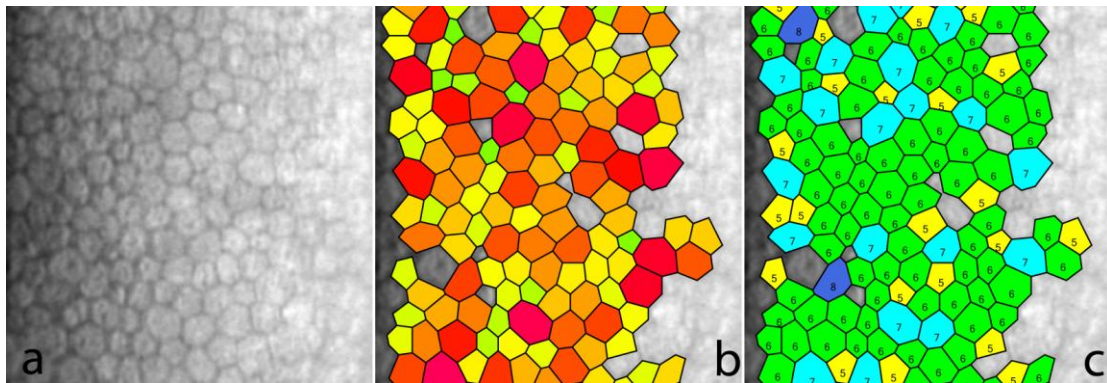
Figure legends:

Figure 1: Example of corneal endothelial cells captured by specular microscopy

A: Endothelial cells – density is 2454 cells/mm²

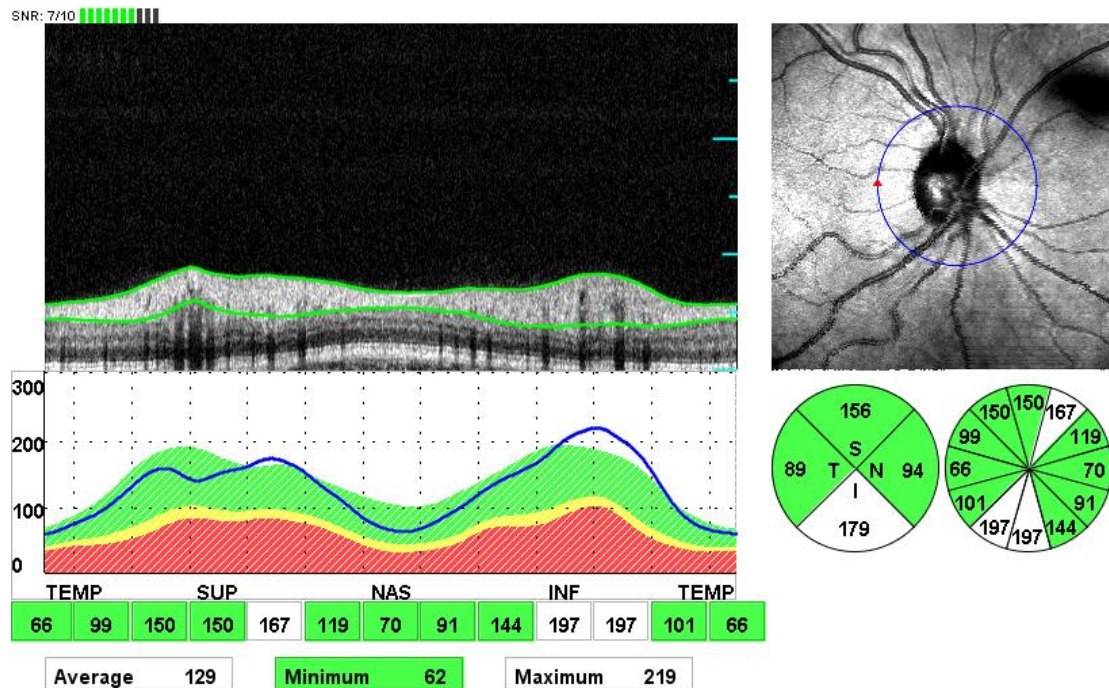
B: Polymegathism assessment – measurement of cell size

C: Pleomorphism assessment – proportion of cells that are hexagonal



Chapter 10

Retinal nerve fibre layer thickness and contrast sensitivity in HIV-infected individuals in South Africa: a case control study



Output from optical coherence tomography providing information about retinal nerve fibre layer thickness

Research paper investigating relationships between contrast sensitivity, the retinal nerve fibre layer and HIV infection in South African adults

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published
 - 2.1.** Where is the work intended to be published? **American Journal of Ophthalmology**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, SD Lawn, HA Weiss, C Cook, CE Gilbert
 - 2.3. Stage of publication – ~~Not yet submitted/~~**Submitted/**~~Undergoing revision from peer reviewers' comments/~~In press

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments and conducted the data collection. I performed all the optical coherence tomography scans and clinical examinations. I performed the statistical analyses with advice from Helen Weiss. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



TITLE PAGE

Retinal nerve fibre layer thickness and contrast sensitivity in HIV-infected individuals in South Africa: a case-control study

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Abstract

Purpose: To investigate the relationships between contrast sensitivity (CS), retinal nerve fibre layer (RNFL) and HIV-1 infection in South African adults.

Design: Case-control study

Methods:

- Setting: Township primary care clinics.
- Study Population: 225 HIV-infected individuals without retinal opportunistic infections [OIs], and 203 gender/age-matched HIV-seronegative individuals.
- Observation Procedures: Peri-papillary RNFL thickness was determined with spectral domain optical coherence tomography in four quadrants. CS was measured using a Pelli-Robson chart.
- Main Outcome Measures: RNFL thickness and CS were evaluated as continuous and binary variables. Multivariable linear and logistic regression were used to assess associations between HIV status and RNFL and CS.

Results: The median age of both groups was similar (41.2 vs. 41.9 years, $p=0.37$). Antiretroviral therapy (ART) was being received by 88% of HIV-infected individuals and their median CD4 count was 468 cells/ μl . Adjusted CS score was lower in HIV-infected participants compared to HIV-seronegative individuals (1.76 vs. 1.82, $p=0.002$). Independent predictors of poor CS in the HIV-infected group were positive frailty status and current HIV viral load >2 log copies/ml. Lower CS score was also associated with thin temporal RNFL in HIV-infected individuals ($p=0.04$). Superior quadrant RNFL thickness was greatest in ART-naïve participants relative to the HIV-uninfected group (p -trend=0.04). Longer ART duration was associated with decreased thickness of inferior and nasal RNFL quadrants (p -trend=0.03 and 0.04, respectively).

Conclusions: Contrast sensitivity is reduced in those with HIV-infection and functionally associated with systemic frailty and unsuppressed viraemia. This may reflect structural changes in the RNFL that are evident despite the absence of OIs.

Abstract word count: 250

MAIN TEXT

Introduction:

In well-resourced settings, antiretroviral treatment (ART) has altered the spectrum of HIV-related eye disease in people living with HIV infection, leading to a lower prevalence of retinal opportunistic infections (OIs) such as cytomegalovirus (CMV) retinitis.^{1, 2} This trend is now becoming apparent in resource-constrained environments where scale-up of ART is being started in individuals with increasingly high CD4 counts. However, abnormalities in visual function, such as reduced contrast sensitivity (CS), altered colour vision and visual field loss also have also been reported in HIV-infected individuals despite effective viral suppression and the absence of retinal OIs. It is thought that these changes may be mediated by an HIV-associated 'neuroretinal disorder' (HIV-NRD) which is characterized by changes in the retinal nerve fibre layer (RNFL).³⁻⁵

One mechanism that may potentially contribute to HIV-NRD is 'accelerated biological' aging that is recognised to be associated with HIV infection.⁶ This refers to conditions which are classically associated with the normal ageing process but which occur at an earlier age in HIV-infected individuals compared with those of similar age who are seronegative.⁷⁻⁹ This may have major implications for long-term morbidity, including for the many millions of people receiving ART in sub-Saharan Africa.^{10, 11} However, there are limited data on visual function (in the absence of ocular OIs) in people living with HIV in this region. Although RNFL changes are strongly related to chronological age,^{12, 13} studies describing visual function, RNFL status and the possibility of HIV-NRD in HIV-infected individuals are needed, particularly as quality of life might be significantly compromised. Indeed, impaired contrast sensitivity (CS) can be more disabling than visual acuity loss.¹⁴

We have recently undertaken a case-control study of adults in South Africa, and have shown that HIV infection is associated with retinal vessel calibre changes consistent with accelerated HIV-related aging and these may reflect an increased risk of cardiovascular disease.¹⁵ We have also shown in the same study population that HIV infection is associated with a functional phenotype consistent with frailty.⁶ This is a clinical syndrome initially described in geriatric populations, characterised by multiple pathologies, low physical activity and slow motor performance.^{16, 17} Frailty predicts cognitive and physical decline and is associated with an increased risk of morbidity and mortality. We now report the findings of visual function from this study, using CS and RNFL as representative measures of ocular aging. The objectives of

this study were to assess differences and identify predictors of CS and RNFL parameters in HIV-infected individuals compared to age-gender matched controls, and to assess whether visual function is related to the phenotype consistent with HIV-related accelerated biological aging that we have already reported.

Methods:

Study participants

HIV-infected participants aged ≥ 30 years were enrolled from a community-based HIV treatment centre in Nyanga district in Cape Town.^{18, 19} Participant recruitment has been reported in detail previously.⁶ In brief, all participants had a confirmed serological diagnosis of HIV and were either about to commence ART (ART-naïve), or were already on first-line ART. Individuals were excluded if they had a history or current ocular OI, which was confirmed from medical case notes. All participants had a best-corrected visual acuity of 20/40 or better in order to be able to perform ophthalmic tests (i.e. CS) satisfactorily. A control group of HIV-seronegative participants was recruited from an HIV clinical prevention trials centre in a neighbouring community by frequency-matching using gender and 5-year age categories.

Data collection

Data on age, sex, weight and height, as well as socio-behavioural factors such as housing, income, smoking and alcohol consumption were collected. Data collected for HIV-infected participants included nadir and current CD4 count, peak and current viral load, staging of HIV according to WHO clinical stage, ART status and regimen. Clinical information was obtained from medical case notes where required. Blood pressure (BP) was measured with a digital sphygmomanometer with a cuff of appropriate size. Mean arterial blood pressure (MABP) was defined as two-thirds of the diastolic plus one-third of the systolic BP.²⁰ Hypertension was defined as a systolic BP of 140mmHg or higher, diastolic BP of 90mmHg or higher, or the combination of self-reported high BP diagnosis and the use of anti-hypertensive medications.²¹ Body mass index (BMI) was defined as weight (in kilograms)/height².

All participants underwent a full ophthalmic examination including measurement of visual acuity, evaluation by slit lamp microscopy and indirect ophthalmoscopy. Contrast sensitivity was measured with a Pelli-Robson chart

(Haag-Streit, Essex, UK). The chart has eight lines of letters with two groups of three letters per line. Testing was performed at 1 m with a 0.75 diopter lens added to the manifest refraction if necessary. The logarithm to the base 10 of the CS measurement was obtained from the Pelli-Robson chart and used in analyses. Investigators who conducted the tests were masked to other data. RNFL thickness was obtained using the Spectral OCT/SLO optical coherence tomography machine (Opko/OTI Inc, Miami, FL). The Spectral OCT/SLO uses a scanning laser diode of 830nm to provide images of ocular microstructures. A peripapillary protocol inbuilt in the software was used to determine the average and quadrant-specific RNFL thickness (superior, inferior, temporal and nasal).

The study was approved by the Ethics Committees of the London School of Hygiene and Tropical Medicine and the University of Cape Town Faculty of Health Sciences, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants

Statistical analyses:

One eye was randomly selected for analysis. Where an eye was not available, for example due to trauma or corneal opacity, the contralateral eye was used. LogCS scores and RNFL were initially analysed as continuous variables. Age and sex-adjusted linear regression was performed to compare logCS score and RNFL parameters respectively by HIV status. A binary categorisation of logCS was defined using the a-priori defined cutpoint of the 25th percentile in the control group to denote 'poor CS' (1.65). Further analyses were undertaken using the cut-off of 1.50 as defined by Shah and associates.²² Similarly, a binary categorisation of RNFL was defined using the cut-off of the 25th centile in the control group to denote 'thin RNFL' (\leq 25th percentile; average 101 μ m, superior 118 μ m, inferior 124 μ m, temporal 62 μ m, nasal 74 μ m). Logistic regression was used to assess predictors of poor CS in those participants on ART. Multivariable linear regression models were used to examine the relationship of RNFL parameters as the dependent variable, with HIV status and other explanatory variables (age group [30-39; 40-49; >50 years], gender, MABP; BMI, smoking) as independent variables. Separate models were used for HIV-infected participants to investigate the effects of HIV-related variables. Marginal adjusted means for RNFL parameters were estimated at the mean value of covariates in the model. The Wald test was used to assess statistical significance of the association of each explanatory variable on RNFL thickness. All analyses were performed with Stata 12 (Stata Corp, College Station, TX).

Results

Participant characteristics:

Characteristics of the 225 HIV-infected individuals and 203 HIV-seronegative age/gender frequency matched HIV-seronegative individuals are reported in Table 1. Although frequency matching was undertaken, some individuals did not have OCT images of adequate quality for analysis, resulting in a greater proportion of HIV-infected participants being included in the analyses. However, those with images did not differ from those without with respect to age, gender or other clinical/demographic characteristics ($p > 0.10$ for all variables; data not shown). HIV-infected participants tended to have a greater income (attributable to a government health-related grant), to be non-smokers, reported lower consumption of alcohol and had a lower BMI than HIV-seronegative individuals. There was little difference in visual acuity between the two groups. Among the HIV-infected participants, 88% of whom were receiving ART, the current median CD4 count was 468 cells/ μ L (interquartile range: [IQR]: 327-607) and 84.9% had an undetectable plasma viral load (VL), defined as < 50 copies/ml.

Contrast sensitivity:

Data on CS were available for 216 HIV-infected individuals and 215 HIV-seronegative individuals. Mean logCS was lower in HIV-infected individuals compared to HIV-seronegative participants (1.77 vs. 1.82, $p = 0.005$). After adjustment for age, sex, smoking status (yes/no), MABP and BMI, mean logCS values still remained lower in HIV-infected individuals (1.76 vs. 1.82, $p = 0.002$). HIV-infected individuals were also more likely to have 'poor CS' (< 1.65) compared to their uninfected counterparts (adjusted proportions: 43.5 vs. 31.8%, $p = 0.01$).

In multivariable analysis among HIV-infected participants on ART, poor CS was associated with positive frailty status (OR 3.04; 95%CI: 1.25-7.35, $p = 0.01$) (Table 2) and HIV viral load > 2 log copies/ml (OR 3.03; 95%CI: 1.02-8.97, $p = 0.05$).

Retinal nerve fibre layer:

Table 3 shows the average and quadrant RNFL thickness stratified by HIV status and by viral load status. Average RNFL thickness was similar between two groups as stratified by HIV status (yes; no). RNFL thickness was also

stratified according to viral load status (Table 3). There was a trend of greater RNFL thickness of the superior quadrant with greater HIV viremia: the mean superior RNFL thickness in HIV-seronegative individuals was 132.2 μ m, compared to 133.8 μ m in HIV-infected individuals on ART with non-detectable VL, and 140.0 μ m in HIV-infected individuals who were ART-naïve (and had detectable VL), p-trend=0.04. A similar trend was observed for the inferior quadrant (p-trend=0.13). Associations of RNFL thickness with current or nadir CD4 count were not observed.

Among HIV-infected participants increasing age, as expected, was associated with thinning of the RNFL. Increasing duration of ART was associated with reduced thickness of the RNFL in nasal and inferior quadrants (Table 4). No association was detected with type of ART. Similarly, average and superior quadrant RNFL thickness was lower in those with advanced HIV infection at ART initiation, as defined by WHO clinical stage 3 or 4.

Relationship between CS and RNFL thickness

Finally, we assessed CS score according to RNFL thickness (thin or normal) in HIV-infected participants. Lower CS was associated with thin temporal RNFL (1.70; thin RNFL, vs. 1.78; normal RNFL, p=0.04). Logistic regression to assess the predictive value of poor CS as a risk factor for temporal thinning of the RNFL showed that the odds ratio using a cut off of 1.65 was not significant (OR: 1.46; 95%CI: 0.64-3.34, p=0.37). However, those with a CS value <1.5 had an almost threefold risk of thin temporal RNFL (OR: 2.78; 95%CI: 1.03-7.54, p=0.04).

Discussion

This study provides clear evidence that HIV infection is strongly associated with poor contrast sensitivity in this South African population. Data from other regions, particularly the USA, demonstrate evidence of an HIV-related 'neuro-retinal disorder', comprising subtle vision abnormalities in the absence of opportunistic infections.^{3, 5, 22, 23} However, this has not been investigated in sub-Saharan Africa to date, where access to ART and the aging HIV-infected population both continue to increase. In a study population from this region we assessed both structural and functional components of the RNFL. Our findings relating to RNFL thickness are novel: higher levels of HIV viremia were associated with increased RNFL thickness, whereas longer duration of ART was associated with decreased RNFL thickness. We also found frailty to be an important predictor of poor CS. These findings have potentially

important implications for long-term visual function particularly among the expanding HIV-infected aging population in sub-Saharan Africa.

We have previously demonstrated that HIV infection is associated with an increased risk of frailty in this South African population, providing evidence that this functional phenotype is associated with HIV-related accelerated aging.⁶ The present study builds on these findings, demonstrating that frailty is an important predictor of poor CS. This suggests that there may be a visual component to frailty in HIV infection. This is further corroborated by the finding that abnormal CS is also independently associated with mortality in individuals with AIDS,²⁴ and may be a sensitive indicator of generalized aging.²⁵ However, the present criteria for frailty assessment do not contain any visual function indicators. In light of these findings, a validation study that includes visual function as another measure of frailty may be warranted. Changes in the RNFL have been demonstrated in conditions with neuro-cognitive decline (e.g. Parkinson's Disease; Alzheimer's Disease)^{26, 27} and a neuro-cognitive component may also contribute to the frailty phenotype,^{28, 29} as well as HIV-associated neurocognitive disorders³⁰. Therefore measurement of RNFL may also be a useful novel predictor in this context.

The frequency of poor CS was markedly higher in HIV-infected individuals compared to controls, yet the median visual acuity between the two groups was the same, and all participants had visual acuity better than 20/40. Contrast sensitivity loss can be present even when visual acuity and fields are relatively intact.³¹ It is also a better predictor of mobility performance than visual acuity.³² These findings highlight that visual complaints from HIV-infected individuals may be related to poor CS, and that visual acuity testing (often performed as a baseline 'screen' by HIV physicians to guide further referral) will often be normal. Assessment of CS could be a useful aid in initial examination of patients with symptoms suggestive of visual dysfunction.

In the context of accelerated aging and HIV, reduced thickness of the RNFL in HIV-infected individuals compared to uninfected counterparts of similar age might be expected as RNFL thickness decreases with increasing chronological age.^{12, 13} Other studies have compared RNFL thickness in HIV-infected individuals with HIV-seronegative controls, but it is difficult to make comparisons as study populations differ in terms of demographic and HIV-related characteristics as well as in the methods used to assess RNFL. Thinning of the RNFL has been detected in HIV-infected participants in the USA with low nadir CD4 count when compared with HIV-infected individuals

with higher nadir CD4 count and HIV-seronegative controls in using OCT.^{4, 33} Significant thinning of the RNFL in HIV-infected participants (regardless of CD4 count) compared to uninfected controls, was observed with confocal scanning laser tomography,³⁴ whereas a study from Brazil did not find a significant difference in RNFL thickness between HIV- infected and uninfected participants using OCT.³⁵ In our study, we did not observe associations related to CD4 count and RNFL thickness, however we did detect associations with HIV viremia. It is therefore difficult to place our study in the context of previous work, as the epidemiology of HIV in sub-Saharan Africa is likely to be different to that in other regions. In addition, the HIV and ART 'trajectory' may be at earlier stages compared to HIV cohorts in well-resourced settings (where the majority of studies have been conducted) thus, our findings may reflect those seen relatively early on in chronic HIV infection.

Our findings suggest that ART duration is an important factor in determining RNFL thickness (inferior and nasal quadrants) after adjusting for age and other co-variates, and we have previously shown that narrower retinal arterioles are associated with increasing duration of ART, independently of age.¹⁵ RNFL thinning and retinal arteriolar narrowing may be related to early vascular dysfunction in the nerve fibre layer, mediated by either HIV infection or ART. Longitudinal studies are required to evaluate the contribution of ART and HIV infection to possible accelerated aging changes in the RNFL.

The association of detectable HIV viremia with an *increase* in RNFL thickness in our study population is biologically plausible. Medzhitov³⁶ used the term "para-inflammation" to describe an intermediate tissue adaptive response: infection and injury lead to full inflammation, whereas chronic tissue stress initiates mild low-grade 'para'-inflammation, a mechanism which attempts to maintain tissue homeostasis and monitor tissue malfunction. However, chronic para-inflammation can lead to disease progression and is thought to be an important process in age-related retinal diseases.³⁷ Increased levels of HIV viremia could initiate a para-inflammatory process in the retina, which may manifest as increased thickness of the RNFL. Kalyani and associates³ have also reported greater RNFL thickness in a subgroup of HIV-infected individuals in the USA. They suggested that mitochondrial toxicity (mediated by HIV or ART) may cause axonal damage to the RNFL, leading to an initial phase of swelling before atrophy. Similar mechanisms are postulated in Leber's hereditary optic neuropathy (LHON), a mitochondrial disorder where mitochondrial dysfunction leads to degeneration of retinal ganglion cells and their axons in the optic nerve.³⁸ OCT measurements in

LHON patients show that RNFL thickness increases in the pre-symptomatic stage, followed by a reduction over time.³⁹ Additionally, we found that lower CS score was associated with thinner RNFL in the temporal quadrant, a finding also demonstrated by Kalyani and associates.³ Anatomically, this could reflect preferential damage to the small-calibre axons of the maculopapillary bundle, similar to proposed mechanisms occurring in LHON.^{3, 38}

A key strength of this study is the inclusion of an age/gender-matched control group with a similar socio-demographic profile as the HIV-infected individuals. By recruiting from the same community, we aimed to reduce the likelihood of differential risk exposure in line with the recommendation for careful study design when investigating premature aging in HIV.⁴⁰ Moreover, this allowed us to directly reference findings in HIV-infected individuals to a comparable control population, rather than recourse to normative data from other populations. As there are few data relating to normative visual function or RNFL in African populations, reference to other ethnic or geographical populations could produce erroneous comparisons. Our study population is also large compared to several studies of RNFL and visual function in HIV, and we used the same OCT machine and operator for the duration of the study to minimize potential variability in measurements, which can occur with different OCT machines and operators.⁴¹

This study had some limitations. The study design means we cannot infer whether HIV is causally related to poor CS function or changes in RNFL, nor can a temporal relationship be established. The measurement of CS is subjective, however we adhered to standardized protocols (e.g. lighting, measurement distance) and used the same chart and examiner in the same location. Another potential limitation is that misclassification of smoking and alcohol consumption may have occurred, with HIV-infected participants wanting to demonstrate 'healthy behaviour' which could have led to confounding. Finally, in the context of further defining the HIV-neuroretinal disorder it would have been ideal to have data on visual fields and colour vision, however, this initial data provide an informative basis from which to plan further studies in this study population or region. Further investigations such as micro-perimetry and retinal electrophysiology would also help to elucidate possible mechanisms and pathways of HIV-associated neuro-retinal disorder.

In summary, HIV-infected individuals in South Africa demonstrate abnormal CS, and changes in RNFL thickness related to viremic status. Longitudinal studies are needed to determine whether changes in RNFL and systemic indicators such as frailty predict change in visual function, and equally importantly, the role of ophthalmic indicators in predicting the biologically aged phenotype in chronic HIV infection.

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b. Financial Disclosures: All authors have completed and submitted the ICMJE form for disclosure of potential conflicts of interest; additionally no financial disclosures were reported.

c. Contributions of Authors: Design of the study (SP, CG, SDL); Conduct of the study (SP, CC); Data collection (SP, CC); Data management (SP), Data analysis (SP, HAW), Interpretation of the data (SP, CG, SDL), Preparation of initial manuscript (SP), Review of manuscript (SP, SDL, HAW, CC CG), Approval of the manuscript (SP, SDL, HAW, CC CG).

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Table 1: Characteristics of study participants

Variable	HIV-infected (225)		HIV-seronegative (203)		P- value	
Age (mean±SE)					0.37	
	N	%	N	%		
Age, years by group						
	30-39	112	49.7	98	48.3	0.88
	40-49	73	32.4	65	32.0	
	>50	40	17.8	40	19.7	
Gender						
	Male	60	26.7	49	24.1	0.55
	Female	165	73.3	154	75.9	
Income						
	<ZAR1000/month	128	56.9	140	69.0	0.01
	>ZAR1000/month	97	43.1	63	31.0	
Smoking status						
	Non-smoker	190	84.4	146	71.9	0.002
	Smoker	35	15.6	57	28.1	
Alcohol consumption						
	No	156	69.3	112	55.2	0.002
	Yes	69	30.7	91	44.8	
Hypertension						
	No	144	64.0	122	60.1	0.41
	Yes	81	36.0	81	39.9	
Body mass index (kg/height ²)			28.0±0.4		31.9±0.6	0.0001
Visual acuity (presenting)(median)			20/20 (20/20-20/25)		20/20 (20/20-20/32)	0.34
HIV-related characteristics (n=225)						
WHO stage						
	1/2	62	27.6			
	3/4	163	72.4			
ART naïve		27	12.0			
CD4 count in ART naïve group (cells/μl)		27	170 (81-201)			
Log ₁₀ VL in ART naïve group (copies/ml)		16	4.79 (4.07-5.09)			
Current CD4 count - ART group (cells/μl)			468 (327-607)			
Nadir CD4 count - ART group (cells/μl)			136 (77-175)			
% with detectable VL in ART group		30	15.1			
Peak Log ₁₀ VL in ART group (copies/ml)			4.47 (3.74-4.97)			
Duration of ART, months		198	56.5 (34-74)			
ART Regimen						
	Containing AZT/3TC	118	59.6			
	Other	80	40.4			

Table 2: Predictors of poor contrast sensitivity among HIV-infected participants on ART (N=190)

Variable	Odds ratio (OR)* For reduced contrast sensitivity	P
Age group 30 years per 10 year increase	1 1.22 (0.75-2.00)	0.43
Sex		
Male	1	
Female	1.73 (0.66-4.57)	0.27
Frailty status		
Not frail	1	
Frail	3.04 (1.25-7.35)	0.01
Smoking status		
Non-smoker	1	
Current	0.49 (0.17-1.45)	0.20
Current CD4 count		
<400 cells/ μ l	1	
>400 cells/ μ l	0.84 (0.40-1.77)	0.64
Nadir CD4 count		
<100 cells/ μ l	1	
100-200 cells/ μ l	1.66 (0.79-3.50)	0.28
>201 cells/ μ l	2.30 (0.69-7.63)	
Current viral load		
<2 log copies/ml	1	
>2 log copies/ml	3.03 (1.02-8.97)	0.05
Peak viral load		
<4.5 log copies/ml	1	
>4.5 log copies/ml	0.85 (0.43-1.68)	0.64
WHO clinical stage		
1/2	1	
3/4	1.84 (0.78-4.32)	0.17
ART duration (months)		
<24	1	
25-48	2.28 (0.74-7.08)	
49-72	2.73 (0.85-8.75)	0.30
>73	1.68 (0.50-5.61)	
ART regimen		
Containing AZT/3TC	1	
Other	1.20 (0.61-2.34)	0.60

*Adjusted for all variables in table, and mean arterial blood pressure and BMI, age group used as a linear term in model

Table 3: RNFL thickness in μm (standard error) by HIV and viral load status – adjusted for age, gender, mean arterial blood pressure, smoking status and BMI:

RNFL Thickness in μm (SE)	HIV- (n=203)	All HIV+ (n=225)	P-value ^a	On ART VL<50 copies/ml (n=168)	On ART VL>50 copies/ml (n=30)	ART-naive (n=27)	P-value ^b
Average**	108.7 (0.9)	109.7 (0.8)	0.41	109.0 (1.0)	111.9 (2.4)	111.3 (2.5)	0.52
Superior	132.2 (1.5)	135.1 (1.4)	0.16	133.8 (1.6)	138.5 (3.8)	140.0 (4.1)	0.04*
Inferior	137.9 (1.5)	135.6 (1.4)	0.27	134.7 (1.7)	137.7 (4.0)	139.5 (4.3)	0.50
Nasal	88.4 (1.5)	91.1 (1.5)	0.19	91.2 (1.7)	89.5 (4.0)	95.1 (4.4)	0.13*
Temporal	72.5 (0.9)	73.1 (0.9)	0.65	73.1 (1.0)	73.3 (2.4)	70.7 (2.7)	0.85

**Limited images in this group; total n=402 (188/160/28/26)

a - for difference between HIV-infected and HIV-seronegative groups

b – for difference between HIV-seronegative and HIV groups by viral load status

* - refers to p-value using test for trend

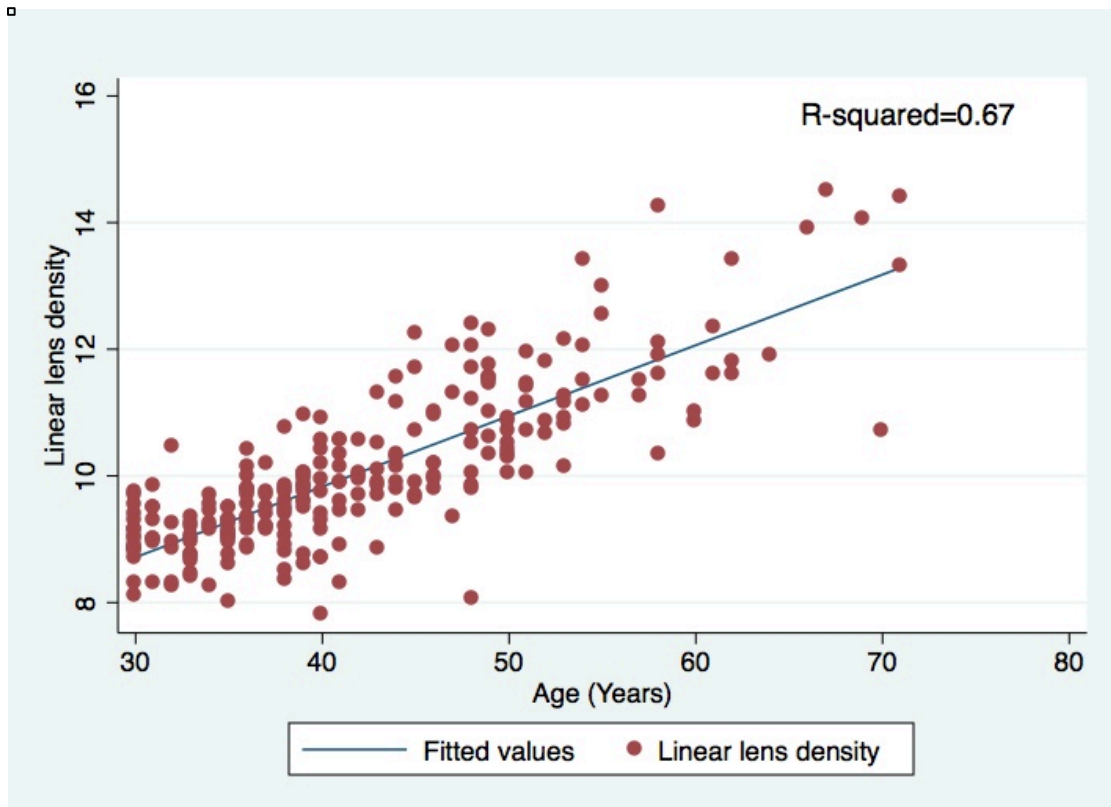
Table 4: RNFL thickness in μm in HIV-infected participants (n=198); *=p-value for test of trend

Parameter	Retinal nerve fibre layer thickness (μm)									
	Average		Superior		Inferior		Nasal		Temporal	
Age group, years										
30-39	112.0		138.3		139.7		89.3		76.6	
40-49	109.5	<0.0001*	133.7	0.004*	133.4	0.001*	93.7	0.29	70.6	0.006*
>50	102.2		124.9		125.2		88.5		69.3	
ART duration, months										
<36	110.4		136.6		138.5		93.0		72.3	
37-72	110.1	0.37*	135.1	0.27*	137.0	0.02*	93.4	0.04*	73.2	0.81
>73	107.7		131.4		128.6		84.1		74.5	
WHO clinical stage										
1/2										
3/4	112.5	0.07	141.1	0.03	139.7	0.14	90.2	0.92	74.7	0.56
	108.7		132.7		133.8		90.7		72.9	
Current viral load										
<2 log copies/ml	109.2		134.1		134.8		91.1		73.0	
>2 log copies/ml	111.8	0.37	137.1	0.58	136.5	0.92	87.5	0.29	75.4	0.47

Adjusted for age, gender, smoking status, mean arterial blood pressure, BMI, nadir and current CD4 count, ART regimen and all parameters displayed in table

Chapter II

Assessment of candidate ocular biomarkers of aging in a South African population: Relationship with systemic biomarkers



Regression line of linear lens density against chronological age in years

Research paper investigating the association of potential ocular biomarkers of aging with systemic biomarkers of aging

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **Mechanisms of Ageing and Development**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, CE Gilbert, SD Lawn, HA Weiss, T Peto, C Cook TY Wong, PG Shiels
 - 2.3. Stage of publication – ~~Not yet submitted~~/**Submitted**/~~Undergoing revision from peer reviewers' comments~~/**In press**

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments with assistance from Clare Gilbert and Paul Shiels. I conducted the data collection and performed all ophthalmic tests. I performed DNA extraction, quantification and RNA preparation on site in Cape Town. I performed the statistical analyses with advice from Helen Weiss. Paul Shiels' laboratory team were responsible for measurement of CDKN2A levels and telomere length. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



Assessment of candidate ocular biomarkers of ageing in a South African adult population: relationship with chronological age and systemic biomarkers

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Suggested running head: Ocular biomarkers of ageing

Abstract:

Certain anatomic and functional parameters of the eye change with increasing chronological age. They may, therefore, serve as potential biomarkers of ageing. We investigated associations between four such ocular parameters (lens density, retinal vessel calibre, corneal endothelial cells and retinal nerve fibre layer thickness) and two 'cellular' biomarkers of ageing (leukocyte telomere length and CDKN2A expression) and with frailty (a clinical correlate of biological ageing) in a population of South African adults. All ocular parameters revealed an association with either telomere length or CDKN2A expression. However, lens density was most strongly correlated with age, increased CDKN2A expression, and with frailty ($p=0.05$ and 0.03 , respectively). Narrow retinal arteriolar diameter, associated with increased chronological age, was also associated with increased CDKN2A expression (0.42 vs. 0.31 , $p=0.02$) but not with frailty. Ocular parameters may aid in determining biological age, warranting investigation in longitudinal studies.

Word count: 141

Keywords: Telomeres; CDKN2A; lens density; retinal vessel calibre; corneal endothelium; retinal nerve fiber layer; frailty

1. Introduction

There is substantial variation in the health and functional status of older populations in many developing countries as well as in developed countries (Lloyd-Sherlock et al., 2012). The reasons for these variations are poorly understood, highlighting the need for translational age-related research within a global context (Salomon et al., 2013; Wang et al., 2013). Chronological age is an imprecise measure of biological ageing, due to inter-individual differences in rates of ageing. The disconnection between chronological age and lifespan has led to a search for effective and validated biomarkers of ageing (BoA), defined as “biological parameters of an organism that either alone or in some multivariate composite will better predict functional capability at some late age, than will chronological age” (Baker and Sprott, 1988).

It is acknowledged that many age-related chronic diseases such as cardiovascular disease and Alzheimer's disease share common pathways of early dysregulation, and that the development of markers and diagnostic techniques is fundamental to understanding healthy biological ageing and thus these diseases (Franco et al., 2007). The need for research on how healthy ageing can be achieved in the context of life-time trajectories has led to concept of the 'Healthy Ageing Phenotype' (Franco et al., 2009). With demonstrable molecular, epigenetic and clinical correlates of ageing, the eye may be a model system for validating potential biomarkers (Pathai et al., 2013).

The unique access to and visibility of ocular tissues and range of visual functions permits investigation of a wide variety of physiological and pathological mechanisms. Many age-related ocular changes also have systemic associations or correlates of ageing in other end-organs or body systems but may be easier and less invasive to measure in the eye (Table 1). For example, changes in the lens, which has an extremely high protein content, may reflect systemic changes in protein structure and function in other organs (Truscott, 2010, 2011; Wormstone and Wride, 2011). Corneal endothelial cell parameters, lens density, retinal vessel calibre and thickness of the retinal nerve fibre layer (RNFL) are ocular parameters that vary with age that can be objectively and non-invasively imaged and assessed.

Ideally, proposed ocular biomarkers should be assessed in relation to established and validated BoA at a clinical or cellular level. Only two validated BoA, telomere length (TL) and CDKN2A expression, have so far been found to satisfy the majority of the criteria proposed by Baker and Sprott (Baker and Sprott, 1988). Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. Their DNA component shortens with somatic cell division and upon reaching a critically short length, a DNA damage signal leads to growth cycle arrest, resulting in replicative senescence (Saretzki and Von Zglinicki, 2002; von Zglinicki, 2002). Telomere shortening is associated with increasing chronological age and several pathologies, including cardiovascular disease (Starr et al., 2007) and renal dysfunction (Carrero et al., 2008). TL may be useful as a composite measure of healthy

ageing, but not as a BoA when used in isolation (Der et al., 2012; von Zglinicki, 2012). Expression levels of the cell cycle regulator CDKN2A may represent a more robust BoA (Shiels, 2010). CDKN2A acts as a tumour suppressor and maintains cells in a state of growth arrest, both in replicative and stress induced-senescence. Increasing levels of CDKN2A transcriptional expression occur with increasing age and decreasing function of solid organs and peripheral blood leucocytes (PBLs) (Koppelstaetter et al., 2008; Krishnamurthy et al., 2004; Liu et al., 2009; McGlynn et al., 2009). However, there are limited data on how these parameters correlate with measures of physical frailty (Woo et al., 2008), a functional state characterised by an increased risk of multiple pathologies, low physical activity and slow motor performance (Fried et al., 2001a). Frailty predicts cognitive and physical decline and is associated with an increased risk of morbidity and mortality, and may therefore act as a 'clinical' biomarker of ageing (Fried et al., 2001a).

There are few data on biological ageing in sub-Saharan Africa, a region where the population of elderly people is rapidly expanding, and where the incidence of age-related non-communicable diseases is steadily increasing (Marquez and Farrington, 2012). The aim of this study was to investigate the association of a variety of ocular candidate BoA with 'systemic' BoA and frailty status in a South African adult population.

2. Methods:

2.1 Study population:

Individuals aged ≥ 30 years from an HIV prevention trials site in a township community of Cape Town, South Africa (Emavundleni Centre, Crossroads) were recruited as HIV-seronegative controls as part a case-control study investigating HIV and ageing (Pathai et al., 2012a; Pathai et al., 2012b). Socio-demographic information and medical history were obtained by interviewing participants in their first language (Xhosa or English). Data collected included factors known to affect ageing (e.g. UV exposure, smoking history). All participants underwent a full ophthalmic examination including measurement of visual acuity, evaluation by slit lamp microscopy and indirect ophthalmoscopy.

The study was approved by the Ethics Committees of the London School of Hygiene and Tropical Medicine and the University of Cape Town Faculty of Health Sciences, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

2.2 Anthropometry, blood pressure and physical function including frailty assessment:

Blood pressure (BP) was measured with a digital sphygmomanometer. Mean arterial blood pressure (MABP) was defined as two-thirds of the diastolic plus one-third of the systolic BP (Wong et al., 2003). Hypertension was defined as a systolic BP of 140mmHg or higher, diastolic BP of 90mmHg or higher, or

the combination of self-reported high BP diagnosis and the use of anti-hypertensive medications (Wong et al., 2005). Body mass index (BMI) was defined as weight (in kilograms)/height².

Physical frailty was defined by the presence of ≥ 3 of 5 criteria: i) unintentional weight loss (self reported and verified from clinic records where possible) ii) self-reported low physical activity, iii) self-reported exhaustion, iv) weak grip strength and v) slow walking time. Pre-frailty was defined as the presence of one or two of these criteria. Detailed information is available in the Supplementary Methods.

2.3 Blood-based biomarkers:

DNA/RNA extraction

DNA was extracted from PBLs using the Maxwell™ Automated Purification System according to manufacturer's instructions (Promega, USA). DNA concentration and purity were quantified by Nanodrop Spectrophotometer (ThermoFisher Scientific, USA). RNA was extracted using Trizol reagent (Invitrogen, UK) following manufacturer's guidelines. DNA/RNA extraction was performed in Cape Town and samples shipped on dry ice to the University of Glasgow.

Telomere length determination

Telomere lengths were determined by QPCR following the method of Cawthon (Cawthon, 2002). Telomere length determination was performed blindly using a Roche Light Cycler LC480. Briefly, telomere length analyses were performed in triplicate for each sample, using a single-copy gene amplicon primer set (acidic ribosomal phosphoprotein, 36B4) and a telomere-specific amplicon primer set (Koppelstaetter et al., 2008). Refer to Supplementary Methods for further detail.

CDKN2A expression determination

Relative quantitative real-time PCR (qRT-PCR) was used to estimate mRNA levels corresponding to the candidate senescence associated gene - CDKN2A. Expression levels were measured against a reference hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene on an ABI Prism(R) 7500 Sequence Detection System. Sequences of human TaqMan™ Primer/Probe sets were designed by Primer Express algorithm (Applied Biosystems, Austin, TX, USA). The comparative threshold cycle method ($\Delta\Delta CT$) (Livak and Schmittgen, 2001) was employed to quantify relative gene expression.

2.4 Ocular biomarkers

The following four ocular parameters were selected (Table 1). Detailed methods are supplied in the Supplementary Methods.

i) Lens density: Pentacam imaging (Oculus, Wetzlar, Germany) was used to obtain "Scheimpflug images" of the lens and to obtain an objective estimate of lens density on a continuous scale. Lens density increases with increasing chronological age.

ii) Retinal vessel calibre: Participants had stereoscopic 30° colour retinal photographs taken under pharmacological pupil dilation with a fundus camera (CF-2; Canon Inc., Tokyo, Japan). Vessel calibre indices were determined in a semi-automated manner using the IVAN computer program (Singapore Eye Research Institute, Singapore) and a standardized protocol described previously (Wong et al., 2004). Narrowing of retinal arterioles is associated with increasing chronological age (Leung et al., 2003; Wong et al., 2003).

iii) Corneal endothelial cell parameters: A non-contact specular microscope was used (SP02, CSO; Florence, Italy). The operator focused and aligned a real-time image of the participant's eye. Endothelial cell parameters were automatically calculated from this image by the microscope software. Endothelial cell density (ECD) decreases with age, whereas the change in cell size (coefficient of variation) increases with age. The proportion of cells with six sides (hexagonality index) decreases with age.

iv) Retinal Nerve Fibre Layer (RNFL): Measured using Spectral OCT/SLO optical coherence tomography (Opko/OTI Inc, Miami, FL) which uses a scanning laser diode of 830nm to provide images of ocular microstructures. A peripapillary (around optic nerve head) protocol inbuilt in the software was used to determine the average and quadrant-specific RNFL thickness (superior, inferior, temporal and nasal). The RNFL becomes thinner with increasing age (Chi et al., 1995; Kanamori et al., 2003).

2.5 Statistical analysis:

One eye was randomly selected for analysis. Where an eye was not available, for example due to trauma or corneal opacity, the contralateral eye was used. Analyses were performed using Stata 12 (Stata Corp, College Station, TX). Clinical and biological data were summarized as the median with interquartile range (IQR) or mean with standard error (SE), as appropriate. Analyses were conducted on log₁₀-transformed values of telomere length and mean CDKN2A expression to satisfy the assumption of normally distributed residuals. Results are displayed back-transformed to the original scale.

Validation of the biomarkers was performed using linear regression models with age in years as a continuous or categorical variable. Ocular biomarker measurements were divided into quartiles.

Univariable linear regression was performed to compare the quartiles of ocular parameters with mean telomere length and CDKN2A expression and frailty status respectively. Multivariable linear regression models were used to examine the relationships of telomere length, CDKN2A expression as the respective dependent variable with ocular biomarker quartiles and frailty status and explanatory variables (age group [30-39; 40-49; >50 years], gender, MABP; BMI, smoking, UV exposure) as independent variables. Marginal adjusted means for telomere length and CDKN2A expression were estimated at the mean value of covariates in the model. The Wald test was used to assess statistical significance of the association of each ocular parameter on systemic biomarker levels.

3. Results

3.1 Participant characteristics and biomarker distributions:

256 participants underwent assessment. Their median age was 40 years (IQR: 35-49) and 25% (n=64) were male. Characteristics of the participants by gender are given in Table 2. Women reported less alcohol consumption and cigarette use than men ($p < 0.0001$ for both). Men had a higher frequency of hypertension and had lower BMI ($p = 0.01$ and $p < 0.0001$, respectively). The number of participants providing data for each parameter varied, as not every participant was able to complete every ophthalmic test or had a blood sample available for analysis. Summary statistics for each biomarker, stratified by gender and age group are displayed in Table S1. For the majority of biomarkers, there was no evidence of gender differences; however for RNFL parameters, the average, inferior and temporal quadrants were thinner in men ($p = 0.01$, $p = 0.0008$ and $p = 0.02$, respectively).

3.2 Validation of blood-based and ocular biomarkers against chronological age:

Blood-based and ocular parameters were validated against chronological age (Table S2). All parameters except retinal venular diameter and the RNFL nasal quadrant were related to chronological age and so these parameters were not analysed further. The association of the prospective biomarkers with chronological age is presented in Supplementary Figures 1a-1o. The R-squared values of the regressions against chronological age were highest for lens density parameters (linear lens density $R^2 = 0.67$); other biomarkers including TL and CDKN2A had R^2 values < 0.10 . All analyses thereafter were adjusted for age, gender and other possible confounding variables related to the parameter of interest.

3.3 Association of ocular parameters with blood-based biomarkers:

Shorter TL was associated with decreasing endothelial cell density (p -trend=0.08). CDKN2A expression was related to increased variation in endothelial cell size in a non-linear fashion ($p = 0.05$) (Table 3). The linear parameter of lens density was most informative (Table 3). CDKN2A expression increased with increased lens density (p -trend=0.05); the 3-D average lens density parameter also displayed a similar trend (p -trend=0.08).

Expression of CDKN2A was associated with changes in the calibre of retinal arterioles ($p = 0.06$) and in AVR ($p = 0.03$) (Table 3), however a linear trend was not detected. When arteriolar diameter was categorised as a binary variable, ('thin' or 'thick', i.e. either side of the median value) CDKN2A expression was greater in those with thin compared to thick retinal arterioles (0.42 vs. 0.31, $p = 0.02$). TL was not related to arteriolar diameter.

In view of the difference in RNFL thickness between genders (Table S1), data were analysed for RNFL by gender. After adjustment, there were no differences between genders (data not shown), thus data for

both men and women combined are presented. Telomere length was informative for the superior quadrant of the RNFL (Table 3), with shorter telomere length associated with thinner RNFL (p-trend=0.05).

3.4 Association of frailty status with blood-based and ocular biomarkers:

There was a significant trend of increased TL with worsening frailty status (p-trend=0.02) (Table 4). CDKN2A expression also increased with frailty status, however this trend was not statistically significant (p-trend=0.12). Among the ocular biomarkers, lens density was the only parameter associated with frailty status, with increased linear lens density related to greater frailty status (p-trend=0.03).

4. Discussion

In this study we compared several ocular parameters with established and validated systemic BoA (TL and CDKN2A expression) as well as frailty, a clinical correlate of ageing. Objective measurement of lens density was the most informative ocular biomarker, with greater lens density associated with increased CDKN2A expression and with increased frailty status. Retinal arteriolar narrowing was also associated with greater CDKN2A expression. In contrast, lower endothelial cell density and thinning of the RNFL were associated with shorter TL. These findings suggest that a range of structural features of the eye, which can be objectively imaged and measured, may reflect different physiological parameters of ageing. These ocular BoA may provide insights into biological age, ageing trajectories and a range of chronic systemic diseases.

Lens density parameters had the strongest association with chronological age compared to the other biomarkers and fulfil the Baker and Sprott criteria (Baker and Sprott, 1988). Lens density was the most informative ocular biomarker in that it was related to CDKN2A expression, a cellular biomarker of ageing, as well as with frailty status, a clinical correlate of systemic ageing. The human lens is considered an ideal tissue for studying macromolecular ageing, and physiological ageing in general, as biochemical mechanisms in lens proteins may reflect ageing processes elsewhere in the body (Eldred et al., 2011; Michael and Bron, 2011; Truscott, 2010, 2011; Truscott and Zhu, 2010; Wormstone and Wride, 2011). Epidemiological research has demonstrated that individuals with cataracts have a significantly higher mortality rate than those without, even after adjusting for known confounders (Wang et al., 2001; West et al., 2000). Crystallins represent the major structural proteins of the lens and are responsible for the refractive power of the lens (Horwitz, 2000). In other tissues crystallins are also involved in several cellular pathways involving the stress response, apoptosis and cell survival at a systemic and ocular level (Andley, 2008). Thus, crystallins are not only involved in regulatory roles within the eye but also play important roles in several other organs, leading to the suggestion that cataract is a 'bio-indicator' for less obvious, more severe age-related disorders (Graw, 2009). The concept of 'lens transparency' as a biomarker of ageing has already been described (Sanders et al.,

2011), and clinical diagnosis of cataract is associated with leukocyte TL. However clinical cataract is generally diagnosed in later years of life, whereas Scheimpflug imaging of lens density provides objective measurements at any given age. Evaluation of lens density across the age spectrum would be essential to evaluate fully the usefulness of the lens as a biomarker of ageing, and testing whether lens transparency is a predictor of mortality or longevity would provide the strongest evidence. However, this approach may be limited in well-resourced settings where surgical extraction of the lens often occurs with minimal lens opacities, rendering the lens unavailable for assessment. However, lens density measurement from early ages until lens extraction may still provide insight into healthy ageing trajectories.

The retina represents a unique location where the microvasculature can be directly and non-invasively visualised. The technique of semi-automated software applied to digital retinal photographs (Wong et al., 2004) is established as a valid and efficient biomarker of systemic vascular disease (Ikram et al., 2006; Wong T and et al., 2006; Wong et al., 2002). Retinal vascular calibre is considered a structural marker of vascular pathology reflecting the interplay of systemic, environmental and genetic factors (Sun et al., 2009). The strong association between increasing age and narrowed retinal vessels has been demonstrated in several study populations (Leung, 2003; Wong, 2003). Small reductions in retinal arteriolar calibre are associated with clinically relevant changes in blood pressure, e.g. a 10-mmHg increase in systolic BP is associated with a 1.1 μm reduction in arteriolar calibre (Ikram et al., 2004). We found that retinal arteriolar narrowing was associated with increased CDKN2A expression, thus the retinal microvasculature reflect senescent microvascular changes. However, retinal vessels can also be affected by systemic pathology e.g. rheumatoid arthritis, smoking and inflammatory diseases (Ikram et al., 2004; Klein et al., 2006; Van Doornum et al., 2011) and may be manifest as a change in retinal vessel calibre. This could affect the measurement of 'true' biological ageing, therefore the lens might be a better model and biomarker of ageing as it is less susceptible to systemic pathology, and therefore representing a true biomarker of ageing and not disease (Simm et al., 2008).

Corneal endothelial cells change morphology and assume an 'aged phenotype' in several chronic systemic diseases (e.g. renal failure, diabetes) (Larsson et al., 1996; Ohguro et al., 1999) suggesting that they may be useful in assessing cellular dynamics of ageing, particularly as measurement is objective and non-invasive. Reduction in the proliferative capacity of corneal endothelial cells is partly mediated by an age-related increase in expression of CDKN2A that functions to hold a cell in a state of growth arrest (Wang et al., 2012). Increased CDKN2A expression was noted in those with the lowest endothelial cell density, however this trend was not significant. In contrast, we found a trend of decreasing endothelial cell density with shorter TL. Evaluation of corneal endothelial cells via specular microscopy may provide a unique way of measuring biological ageing at a cellular level.

Thinning of the RNFL is associated with older age (Chi et al., 1995; Kanamori et al., 2003), manifest functionally as deficits in colour vision and contrast sensitivity. Our findings of thinner superior RNFL associated with shorter TL are in alignment with data from individuals with age-related neurocognitive disease. Thinning of the superior RNFL in Alzheimer's disease has been observed (Lu et al., 2010; Paquet et al., 2007) and similar findings have been noted in Parkinson's disease and spinocerebellar ataxias (Hajee et al., 2009; Pula et al., 2011). OCT measurement of the RNFL is a quick non-invasive procedure, of importance for patients with cognitive impairment. The retrograde loss of nerve fibre layer tissue in the retina and optic nerve may be an early biomarker of Alzheimer's disease, and possibly the earliest sign of disease, prior to damage to the hippocampal region that impacts memory (Valenti, 2011). Thus RNFL analysis may be best suited in detection of early neurocognitive decline as a marker of 'neurobiological ageing'.

In relation to frailty and TL, a study (Woo et al., 2008) showed no correlation between TL and frailty index. Indeed, women had higher frailty scores and longer TLs. We also detected longer TL with increasing frailty status which is not intuitive, as shorter telomeres would be expected. One possible explanation for this is that induction of stress induced premature senescence (SIPS) (Shay and Wright, 2000), leads to acute growth arrest (in contrast to gradual replicative senescence). This may occur in frailty by a non-quantified factor (e.g. genetics, environment, lifestyle) acting on the affected cell population. Our finding that CDKN2A expression does not follow a similar trend is supportive of such a hypothesis. This would then be expected to result in longer TLs in cells under SIPS contributing to frailty. An alternative explanation is a 'survivor effect' i.e. individuals with poor biological ageing may die earlier, thus participants comprise survivors who have different biological characteristics to non-survivors, accounting for frail 'survivors' having longer telomeres. The relationship between the functional phenotype of frailty (reflecting changes in multi-organ systems) and cellular senescence represents two extremes of biological ageing likely influenced by several external factors, thus a well-defined relationship between these parameters maybe unlikely in any case. Lens density was the only ocular parameter to be associated with frailty, reinforcing its potential role as a biomarker of ageing with associations with cellular BoA as well as the clinical presentation of frailty.

This study has some limitations. Study participants were matched by age, gender and socio-economic status to HIV-infected individuals as part of a case-control study, and are not therefore representative of the general population in South Africa. For example, the gender composition of participants was three-quarters female, reflective of the HIV epidemic in Africa, but not representative of the South African population. There were also differences between genders (smoking, alcohol consumption) that may truly exist or may have been misclassified (e.g. misreporting true smoking habits) and this could have confounded associations of the ocular parameters with the other

biomarkers. Participants were recruited from a community of considerable socio-economic deprivation, and therefore likely to have been exposed to factors known to increase biological ageing such as high UV exposure from outdoor work. Therefore, our data might over-estimate associations related to ageing. Lastly, as study participants are of African ancestry, our results are generalizable to the African population.

In conclusion, our study suggests that the eye has a useful and valid contribution to make in the assessment of biological age. The non-invasive and objective nature of the techniques is an added benefit. Our data suggest that assessment of retinal vessel calibre and lens density may be most informative. Further studies could involve development of an 'ocular ageing index' using ocular parameters to predict not only visual morbidity (visual impairment/blindness), but also systemic morbidity and mortality. RNFL parameters may be useful in developing an index for age-related neurocognitive decline, whereas endothelial cell parameters may aid in understanding cellular mechanisms of senescence. In resource-constrained settings access to facilities and personnel capable of measuring biomarkers extracted from PBLs is likely to be limited. Thus, ocular biomarkers in parallel with more easily measurable systemic biomarkers (e.g. frailty status, blood counts) to assess biological ageing may be more feasible in these environments. Finally, we have proposed a research agenda to further define and validate ocular biomarkers of ageing (Pathai et al., 2013). Longitudinal studies in different populations are needed to assess how ocular parameters change over time in relation to blood-based biomarkers and to other candidate biomarkers that have been previously evaluated (Martin-Ruiz et al., 2011; Simm et al., 2008). These parameters could also be measured in longitudinal evaluation of the 'healthy ageing phenotype' from early adult life onwards to characterise the development of biological capital and ageing trajectories in terms of a 'life course' approach (Kuh, 2007) leading to an improvement in our understanding of how to achieve healthy ageing in societies with rapidly increasing ageing populations.

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Table 1: Biomarkers of aging, methods of measurement and the impact of aging

Anatomical site	Parameter	Method of measurement	Age-related changes
Peripheral blood leukocytes	Telomere length (TL)	qPCR	TL shortens
	CDKN2A expression	qRT-PCR to estimate mRNA levels	Increased expression
Corneal endothelium	Endothelial cell density (ECD) Coefficient of variation (CV) Hexagonality index (Ex)	Specular microscopy	Decreased ECD Increased CV Decreased Ex
Lens	Lens opacity Linear value Peak 3-D average	Pentacam – lens densitometry	All increase
Retina	Retinal nerve fibre layer (RNFL) thickness (Average, superior, inferior, nasal, temporal quadrants)	Optical coherence tomography (OCT)	Thinner RNFL- all quadrants
	Retinal vessel caliber	Semi-automated retinal analysis software applied to fundus photographs	Reduced diameter of arterioles and arterio-venous ratio (AVR)
Systemic	Frailty status Non-frail (no criteria) Pre-frail (1-2 criteria) Frail: ≥3 of 5 criteria	Assessment of walking speed, grip strength, self-report of weight loss, exhaustion and low physical activity	Frailty status increases

Table 2: Characteristics of study participants, n=256

Variable	Male (N=64) N (%)	Female (N=192) N (%)	P
Age (mean ± SE)	42.0±1.00	42.8±0.7	0.61
Median age, IQR	41 (35-48)	40 (35-49)	0.96
Age, years by group			
30-39	27 (42.2)	91 (47.4)	
40-49	28 (43.7)	56 (29.2)	
>50	9 (14.1)	45 (23.4)	0.07
Education			
Did not complete high school	15 (23.4)	32 (16.7)	
Completed high school	49 (76.6)	160 (83.3)	0.23
Income			
<ZAR1000/month	41 (64.1)	133 (69.3)	
>ZAR1000/month	23 (35.9)	59 (30.7)	0.44
Location of work			
Outdoors or grant-holder	44 (68.8)	140 (72.9)	
Indoors	20 (31.2)	52 (27.1)	0.52
Housing			
Formal	38 (59.4)	114 (59.4)	
Informal	26 (40.6)	78 (40.6)	0.99
Shared WC			
No	19 (29.7)	52 (27.1)	
Yes	45 (70.3)	140 (72.9)	0.69
Number of people in household			
Up to 5	40 (72.7)	125 (68.7)	
≥6	15 (27.3)	57 (31.3)	0.57
Smoking status			
Non-smoker	23 (35.9)	162 (83.4)	
Smoker; <10 years	16 (25.0)	17 (8.9)	
Smoker >10 years	25 (39.1)	13 (6.8)	<0.0001
Alcohol			
Nil	14 (21.9)	128 (66.7)	
<1L/week	14 (21.9)	43 (22.4)	
>1L/week	36 (56.2)	21 (10.9)	<0.0001
Hypertension			
No	36 (56.3)	141 (73.4)	
Yes	28 (43.7)	51 (25.6)	0.01
BMI			
<20	14 (21.9)	4 (2.1)	
20-24.9	29 (45.3)	17 (8.9)	
25.0-29.9	13 (20.3)	42 (21.9)	
>30	8 (12.5)	129 (67.2)	<0.0001
Tuberculosis status			
No history	52 (81.3)	173 (90.1)	
Previous history	12 (18.7)	18 (9.4)	
Current	0	1 (0.5)	0.12

Table 3: Association of ocular parameters with blood-based biomarkers

Endothelial cell parameters	Telomere Length			CDKN2A Expression		
	N	Rel T/S	P	N	Mean	P
ECD^a quartiles cells/mm²						
1 st (1952-2461)	59	1.06 (0.99-1.14)		47	0.42 (0.33-0.53)	
2 nd (2466-2612)	59	1.03 (0.96-1.10)		52	0.32 (0.25-0.40)	
3 rd (2617-2798)	58	1.10 (1.03-1.18)		54	0.36 (0.29-0.45)	
4 th (2812-3391)	60	1.14 (1.06-1.22)	P-trend 0.08	51	0.36 (0.29-0.46)	0.30
CV^b quartiles						
1 st (26-32)	66	1.03 (0.97-1.10)		47	0.35 (0.28-0.38)	
2 nd (33-35)	70	1.08 (0.99-1.15)		52	0.31 (0.25-0.38)	
3 rd (36-38)	49	1.13 (1.04-1.22)		54	0.49 (0.38-0.64)	
4 th (39-57)	51	1.10 (1.02-1.19)	0.33	51	0.36 (0.28-0.46)	0.05
Ex^c quartiles						
1 st (24-46)	67	1.13 (1.06-1.20)		57	0.36 (0.29-0.45)	
2 nd (47-50)	59	1.05 (0.98-1.13)		61	0.40 (0.32-0.51)	
3 rd (51-54)	60	1.08 (1.00-1.15)		40	0.35 (0.28-0.44)	
4 th (55-70)	50	1.06 (0.98-1.14)	0.50	46	0.33 (0.25-0.42)	0.66
Lens density**						
Linear quartiles						
1 st (7.8-9.2)	62	1.01 (0.94-1.10)		51	0.29 (0.22-0.38)	
2 nd (9.25-9.8)	59	1.08 (1.00-1.17)		53	0.31 (0.24-0.40)	
3 rd (9.85-10.75)	58	1.14 (1.06-1.22)		50	0.37 (0.29-0.47)	
4 th (10.8-14.5)	61	1.09 (0.99-1.19)	P-trend 0.22	54	0.50 (0.37-0.68)	P-trend 0.05
Peak quartiles						
1 st (10.65-15.35)	61	0.93 (1.00-1.16)		57	0.36 (0.28-0.45)	
2 nd (15.4-18.0)	58	1.07 (1.00-1.15)		50	0.33 (0.26-0.41)	
3 rd (18.05-20.5)	61	1.10 (1.03-1.18)		51	0.37 (0.29-0.46)	
4 th (20.6-52.1)	60	1.07 (0.99-1.15)	0.92	50	0.39 (0.30-0.51)	0.81
3D-average quartiles						
1 st (8.3-9.1)	58	1.01 (0.92-1.09)		52	0.28 (0.21-0.37)	

2 nd (9.15-9.75)	64	1.08 (1.00-1.16)		54	0.39 (0.31-0.50)	
3 rd (9.8-10.85)	57	1.14 (1.06-1.23)		50	0.33 (0.26-0.43)	
4 th (10.9-19.7)	61	1.10 (1.00-1.20)	P-trend 0.16	52	0.46 (0.34-0.62)	P-trend 0.08
Retinal vessels						
Retinal arteriolar quartiles (μm)						
1st (102.62-150.30)	57	1.05 (0.98-1.13)		48	0.40 (0.31-0.51)	
2nd (150.36-161.09)	59	1.13 (1.05-1.21)		50	0.43 (0.34-0.54)	
3rd (161.35-172.72)	60	1.02 (0.95-1.09)		55	0.28 (0.22-0.35)	
4th (172.74-209.71)	61	1.10 (1.03-1.19)	0.15	53	0.34 (0.27-0.44)	0.06
RNFL Quartiles (μm)						
Average						
1st (65-101)	49	1.06 (0.99-1.15)		45	0.42 (0.33-0.54)	
2nd (102-109)	48	1.10 (1.02-1.18)		44	0.28 (0.22-0.36)	
3rd (110-117)	45	1.11 (1.03-1.20)		40	0.30 (0.24-0.39)	
4th (118-149)	46	1.07 (1.00-1.16)	0.84	46	0.40 (0.31-0.51)	0.07
Superior						
1st (61-118)	48	1.02 (0.95-0.10)		41	0.27 (0.21-0.35)	
2nd (119-131)	50	0.99 (0.94-1.09)		43	0.39 (0.31-0.50)	
3rd (132-146)	53	1.11 (0.96-1.20)		48	0.32 (0.25-0.40)	
4th (147-199)	46	1.11 (0.98-1.20)	P-trend 0.05	43	0.40 (0.32-0.52)	0.09
Inferior						
1st (78-124)	48	1.03 (0.95-1.11)		41	0.33 (0.26-0.43)	
2nd (125-138)	51	1.07 (0.99-1.15)		43	0.34 (0.27-0.44)	
3rd (139-151)	50	1.08 (1.00-1.17)		48	0.33 (0.26-0.43)	
4th (151-201)	48	1.07 (0.99-1.16)	0.80	43	0.36 (0.28-0.47)	0.97
Temporal						
1st (38-62)	47	1.04 (0.96-1.12)		41	0.31 (0.24-0.40)	
2nd (63-71)	53	1.07 (1.00-1.15)		51	0.32 (0.26-0.40)	
3rd (72-81)	48	1.10 (1.02-1.19)		43	0.42 (0.33-0.53)	
4th (82-131)	49	1.04 (0.97-1.12)	0.73	40	0.34 (0.26-0.43)	0.33

Footnote to Table 3:

Endothelial parameters: Adjusted for age, gender, smoking, UV exposure, income

a: ECD: endothelial cell density - lowest quartile denotes aged phenotype

b: coefficient of variation – i.e. difference in cell shape; highest quartile denotes aged phenotype

c: Hexagonality index – i.e. proportion of cells that are hexagonal; lowest quartile denotes aged phenotype

Lens density: Adjusted for age, gender, smoking, UV exposure

** -measured on a continuous scale 0-100, 100 being an opaque (completely dense) lens; 4th quartile denotes aged phenotype for all lens parameters

Vessels: Adjusted for age, gender, smoking, BMI, hypertension and venular retinal calibre; 1st quartile denotes aged phenotype

RNFL: Adjusted for age, gender, smoking, BMI, mean arterial blood pressure; 1st quartile denotes aged phenotype

Table 4: Association of frailty with systemic and ocular biomarkers of aging

Biomarker	Not-frail	Pre-frail	Frail	P
Telomere length (N)	89	127	34	
Rel T/S	1.01 (0.96-1.08)	1.09 (1.04-1.14)	1.17 (1.06-1.29)	P-trend 0.02
CDKN2A (N)	75	110	32	
Mean expression	0.32 (0.26-0.39)	0.36 (0.31-0.42)	0.44 (0.32-0.60)	P-trend 0.12
Ocular parameter				
Lens density (N)	92	123	31	P
Scale: 0-100				
Linear	9.94 (9.77-10.13)	10.10 (9.94-10.25)	10.38 (10.05-10.71)	P-trend 0.03
Peak	18.82 (17.78-19.85)	18.68 (17.81-19.54)	18.86 (17.01-20.71)	0.97
3D-Average	10.02 (9.81-10.24)	10.14 (9.95-10.31)	10.31 (9.92-10.70)	P-trend 0.22
Vessel calibre (N)	92	120	31	
µm				
CRAE	160.13 (156.56-163.70)	160.99 (157.97-164.01)	166.30 (159.87-172.72)	P-trend 0.19
AVR	0.60 (0.58-0.61)	0.60 (0.58-0.61)	0.61 (0.58-0.64)	0.64
Endothelial cell parameters (N)	91	121	31	
ECD ^a	2637 (2580-2695)	2587 (2539-2635)	2675 (2572-2779)	0.18
cells/mm ²				
CV ^b	35.1 (34.1-36.1)	35.3 (34.4-36.1)	36.9 (35.1-38.8)	P-trend 0.18
Ex ^c	49.9 (48.6-51.3)	50.3 (49.1-51.4)	47.6 (42.3-50.0)	0.15
RNFL parameters (N)	75	104	24	
µm				
Average	109.9 (106.7-113.2)	108.9 (105.6-110.6)	106.9 (101.4-112.4)	P-trend 0.31
Superior	131.1 (126.2-136.1)	133.4 (129.3-137.4)	130.6 (121.5-139.6)	0.73
Inferior	137.6 (132.6-142.6)	138.2 (134.1-142.3)	137.3 (127.2-146.5)	0.97
Temporal	72.3 (69.1-75.4)	72.6 (70.1-75.2)	72.2 (66.4-77.9)	0.98

Adjusted for age, gender, socio-economic status, smoking, alcohol consumption and TB status

a: ECD: endothelial cell density - lowest quartile denotes aged phenotype

b: CV: coefficient of variation – i.e. difference in cell shape; highest quartile denotes aged phenotype

c: Hexagonality index – i.e. proportion of cells that are hexagonal; lowest quartile denotes aged phenotype

Supplementary Methods

Telomere length determination:

Quality control parameters for the amplifications comprised a cut off of 0.15 for the standard deviation (SD) of the threshold cycle (Ct) for sample replicates. At a SD above 0.15 the sample was reanalysed. The average SD across plates was 0.05. Relative telomere length was estimated from Ct scores using the comparative Ct method after confirming that telomere and control gene assays yielded similar amplification efficiencies. This method determines the ratio of telomere repeat copy number to single copy gene number (T/S) ratio in experimental samples relative to a control sample DNA. This normalised T/S ratio was used as the estimate of relative telomere length (Relative T/S). The inter-assay variation was assessed by comparing the relative telomere estimates (T/S ratio) estimates across assays for the positive controls, assayed on every assay plate. The average inter-assay coefficient of variance was 0.6% for telomere length and 0.23% for 36B4.

Frailty determination

All of these five components described in the original phenotype by Fried et al (Fried et al., 2001b) were used to determine the presence of frailty. However, we used the proxy described by Önen et al (Onen et al., 2009) for the physical activity measure (see Table 1 below for description). Grip strength of the dominant hand was measured three times using a grip dynamometer (Jamar Plus+ Digital Hand Dynamometer, Jamar, US). The average of three weight measurements was recorded in kilograms (kg) to one decimal point. Walking time was assessed using the method of Cesari et al (Cesari et al., 2005). The average of two trials (in m/s) was used for analysis. Participants were excluded from the determination of grip strength if they had pain or arthritis of the dominant hand, and excluded from the walking test if they had paralysis of an extremity or side of the body, or needed to use a walking aid.

Lens density measurement:

Following pupil dilatation with 1% tropicamide, two lens density measurements were made on each eye, and the mean value calculated. The instrument automatically calculated the quality and reliability of a captured image. If an image was found to be of poor quality (i.e. not 'OK' on the image quality specification), the measurement was repeated. Reproducibility of the lens density evaluation in two scans was performed for 50 eyes. Lens densitometry output values were extracted from the image captures in a masked fashion. Image section 90-270 degrees was used for the right eye and image section 270-90 degrees was used for the left eye (Kirkwood et al., 2009). All of the densitometry metrics available from the scan (linear, peak and 3D) were analysed as they reflect different parameters of the lens. The lens density output was presented on a continuous scale from 0 (transparent) to 100 (fully opaque).

Retinal vessel measurement

All participants had stereoscopic 30 degree colour retinal photographs taken of both eyes under pharmacological pupil dilation with a fundus camera (model CF-2; Canon Inc., Tokyo, Japan). Images were centred on the optic disc. Vessel calibre indices were determined in a semi-automated manner using the IVAN computer program (Singapore Eye Research Institute, Singapore) using a standardized protocol described previously (Wong et al., 2004). In summary, the 6 largest arterioles and venules in a ring-shaped area located between 0.5 and 1.0 disc diameter from the optic disc margin were identified (Figure 1). Computer software measured the calibre of these individual vessels, then combined them into 2 summary variables for the eye: the projected calibre size of the central retinal artery (central retinal artery equivalent [CRAE]), and the projected calibre size of the central retinal vein (central retinal vein equivalent [CRVE]), using formulas derived by Parr and Spears (Parr and Spears, 1974a, b) and Hubbard (Hubbard et al., 1999), with revision by Knudtson (Knudtson et al., 2003). A retinal photograph was considered ungradable if eyes had <4 acceptable measurements of either vessel type. The intergrader and intragrader grading reliabilities were assessed using a random subsample of 100 photographs reviewed four weeks after the initial grading. The intra- and intergrader intraclass correlation coefficients ranged from 0.71 to 0.93. Retinal arteriolar and venular calibre are highly correlated, and to account for potential confounding we adjusted for the fellow vessel in multivariable analyses (i.e. adjustment for arteriolar calibre in analyses of venular calibre and vice versa) (Liew et al., 2006).

Table 1: Frailty criteria: adapted from Fried et al.(Fried et al., 2001b) and Önen et al.(Onen et al., 2009)

Criteria	Definition			
Unintentional weight loss	>10 pounds weight loss documented in last year or ≥5% of previous year's body weight			
Low physical activity*	Participants answering 3 when asked whether their health limits vigorous activities such as running, lifting heavy objects 1= not at all, 2 = yes, limited a little or 3 = yes, limited a lot			
Exhaustion	Participants answering 2 or 3 to either one of two statements – “How often have you felt that:” a) Everything you did was an effort or b) I could not ‘get going’ 0 = rarely (<1 day), 1= some of the time (1-2 days), 2 = occasionally (3-4 days) or 3 = most of the time (5-7 days)			
Weak grip strength	Male BMI kg/m ²	Kg	Female BMI kg/m ²	Kg
	≤24	≤29	≤23	≤17
	24.1-26.0	≤30	23.1-26.0	≤17.3
	26.1-28.0	≤30	26.1-29	≤18
>28	≤32	>29.0	≤21	
Slow walking time	Male height (cm)	Seconds	Female height (cm)	Seconds
	≤173			
	>173	≥7	≤159	≥7
		≥6	>159	≥6

*Estimation of physical activity adopted from Önen et al.(Onen et al., 2009); the estimation of physical activity described in the original phenotype used a weighted score of kilocalories expended

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**Table S1: Median values of candidate biomarkers by gender and age group
P-value for difference between genders**

Measure	Age group, years	N	Men		N	Women		P-value
			Median	IQR		Median	IQR	
Systemic biomarkers								
Telomere length Rel T/S	Overall	60	1.04	0.86-1.29	190	1.09	0.91-1.32	0.62
	30-39	26	1.07	0.93-1.29	90	1.13	0.94-1.30	
	40-49	25	1.15	0.92-1.30	55	1.10	0.94-1.34	
	>50	9	0.96	0.57-1.17	45	0.99	0.88-1.29	
	Overall	54	0.43	0.20-0.67	163	0.37	0.20-0.59	
CDKN2A expression	30-39	25	0.29	0.17-0.60	73	0.28	0.15-0.49	
	40-49	21	0.44	0.19-0.72	49	0.52	0.30-0.80	
	>50	8	0.46	0.27-0.95	41	0.40	0.21-0.69	
	Overall	59	9.8	9.2-10.8	187	9.8	9.2-10.8	0.89
Linear	30-39	26	9.3	9.0-9.7	91	9.3	8.9-9.6	
	40-49	24	9.9	9.5-11.1	54	10.3	9.8-11.0	
	>50	9	11.5	10.9-12.4	42	11.3	10.7-12.11	
	Overall	59	17.7	15.4-19.3	187	18.2	15.4-20.8	
	Peak	30-39	26	17.2	13.6-19.2	91	16.2	14.8-18.7
40-49		24	18	15.6-19.0	54	18.8	15.7-20.1	
>50		9	20.3	16.8-23.4	42	22.4	19.6-24.8	
Overall		59	9.8	9.1-10.7	187	9.8	9.1-10.9	0.77
3-D average	30-39	26	9.1	8.8-9.6	91	9.2	8.8-9.6	
	40-49	24	9.9	9.5-10.8	54	10.2	9.7-11.1	
	>50	9	11.6	10.7-12.7	42	11.4	10.8-12.6	
	Overall	59	161.49	148.11-173.6	184	160.95	150.95-172.59	0.91
CRAE	30-39	26	167.03	156.15-175.63	91	160.81	151.01-171.43	
	40-49	25	159.12	140.14-173.3	54	158.08	146.40-169.72	
	>50	8	158.9	152.67-168.5	39	167.90	155.58-177.18	
	Overall	59	273.00	258.59-287.24	184	267.74	258.17-279.53	
	CRVE	30-39	26	273.10	258.95-287.53	91	270.15	258.92-278.53
40-49		25	278.88	256.62-292.41	54	265.68	254.25-279.92	
>50		8	269.17	256.92-274.30	39	270.51	258.06-284.57	

Endothelial cell parameters								
ECD	Overall	59	2646	2461-2784	183	2582	2458-2812	0.85
	30-39	25	2715	2546-2861	91	2602	2448-2894	
	40-49	25	2662	2315-2778	52	2650	2534-2788	
	>50	9	2561	2211-2802	40	2526	2382-2747	
	CV	Overall	59	35	32-37	183	35	32-38
	30-39	25	35	31-38	91	35	31-37	
	40-49	25	34	32-37	52	36	33-39	
	>50	9	36	35-38	40	36	33-40	
Ex	Overall	59	49	47-53	183	50	45-54	0.97
	30-39	25	50	48-53	91	51	46-55	
	40-49	25	49	48-54	52	49	44-54	
	>50	9	48	42-52	40	49	44-52	
	RNFL thickness, μm							
Average	Overall	42	104	96-112	146	110	102-120	0.01
	30-39	18	110	98-118	72	113	107-121	
	40-49	17	103	98-111	42	106	99-115	
	>50	7	96	95-101	32	105	98-116	
	Superior	Overall	49	130	115-147	154	132	121-146
30-39		20	132	116-149	78	139	123-149	
40-49		21	133	116-146	44	129	121-141	
>50		8	116	102-138	32	128	116-146	
Inferior		Overall	48	131	111-143	154	140	127-154
	30-39	20	141	115-150	78	148	133-157	
	40-49	20	127	110-140	44	136	124-149	
	>50	8	127	105-137	32	136	123-144	
	Nasal	Overall	49	86	63-101	154	89	76-103
30-39		20	97	69-109	78	85	71-102	
40-49		21	84	61-94	44	91	75-107	
>50		8	85	51-86	32	88	81-102	
Temporal		Overall	49	69	60-74	154	73	63-82
	30-39	20	67	59-73	78	78	70-85	
	40-49	21	70	65-76	44	68	61-80	
	>50	8	66	54-84	32	67	59-81	
	Frailty status							
Non-frail	Overall	22	34.4		70	36.4		0.29
	30-39	11	40.7		48	52.8		

	40-49	9	32.1	18	32.1	
	>50	2	22.2	4	8.9	
Pre-frail	Overall	39	60.9	91	47.4	0.28
	30-39	16	59.2	40	44.0	
	40-49	17	60.7	28	50.0	
	>50	6	66.7	23	51.1	
Frail	Overall	3	4.7	31	16.2	0.47
	30-39	0	0	3	3.3	
	40-49	2	7.1	10	17.9	
	>50	1	11.1	18	40.0	

Table S2: Regression coefficients of biomarkers with chronological age in years

Biomarker	N	Coefficient	R-squared	P-value
Telomere length	250	-0.0015	0.02	0.05
CDKN2A	217	0.006	0.02	0.02
<i>Lens density</i>				
Linear	246	0.11	0.67	<0.0001
Peak	246	0.30	0.25	<0.0001
Average 3D	246	0.12	0.63	<0.0001
<i>Retinal vessel calibre</i>				
Arteriolar diameter	243	40-49 years: -4.45 >50 years: 4.15	0.03	0.02
Venular diameter	243	-0.02	0.0001	0.86
<i>Endothelial cell parameters**</i>				
ECD	242	-5.40	0.04	0.003
CV	242	0.06	0.01	0.07
Ex	242	-0.14	0.04	0.001
<i>Retinal nerve fibre layer thickness</i>				
Average	188	-0.39	0.07	<0.0001
Superior	203	-0.40	0.03	0.02
Inferior	202	-0.51	0.04	0.003
Nasal	203	-0.03	0.0001	0.88
Temporal	203	-0.36	0.06	0.003

Linear regression models using age in years as a continuous variable in years or as a categorical variable for retinal vessel parameters

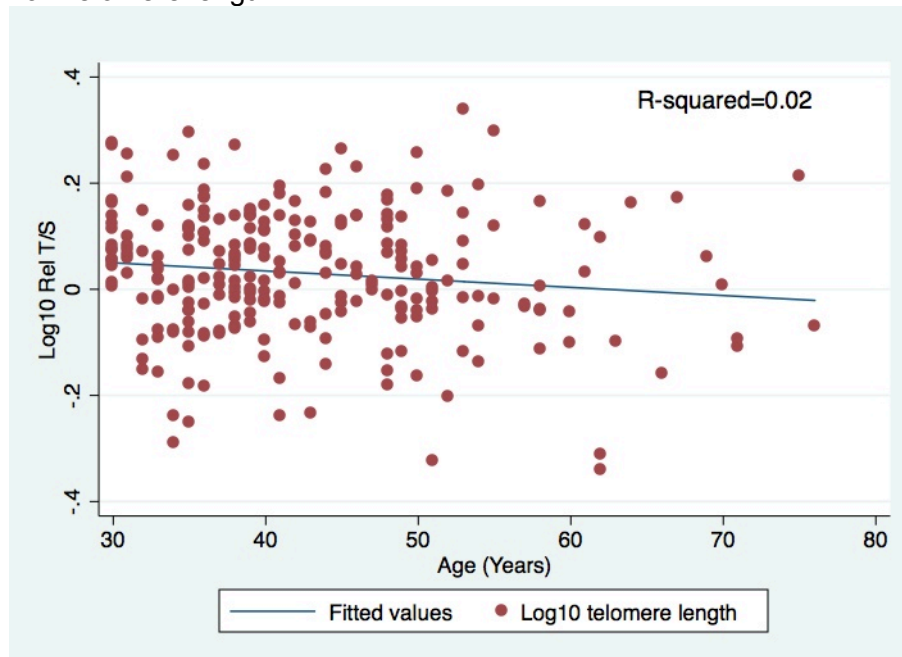
**ECD: endothelial cell density - lowest quartile denotes aged phenotype

CV: coefficient of variation – i.e. difference in cell shape; highest quartile denotes aged phenotype

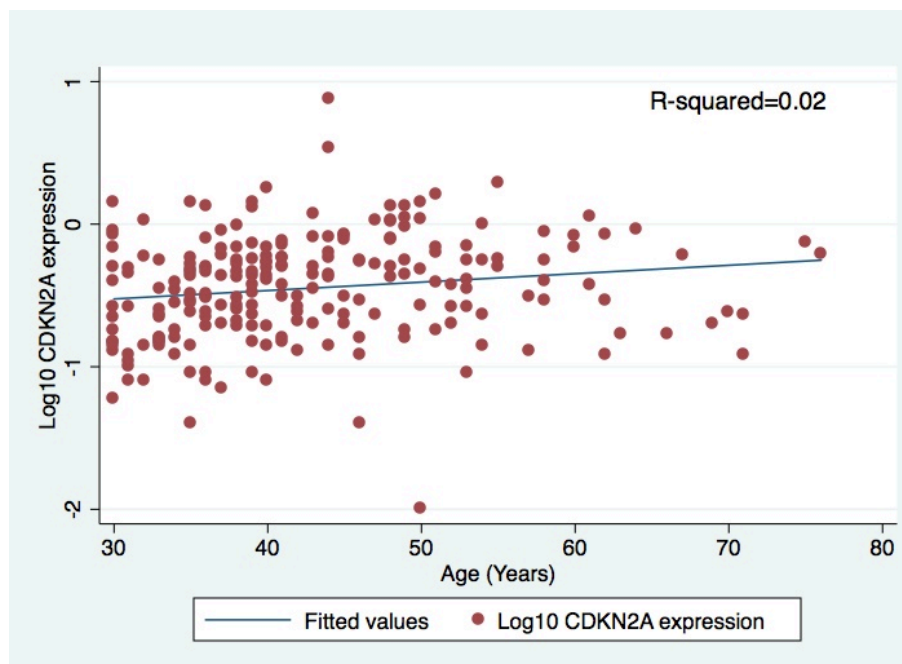
Ex: Hexagonality index – i.e. proportion of cells that are hexagonal; lowest quartile denotes aged phenotype

Supplementary Figures – Scatter plots of biomarkers: regression with chronological age in years

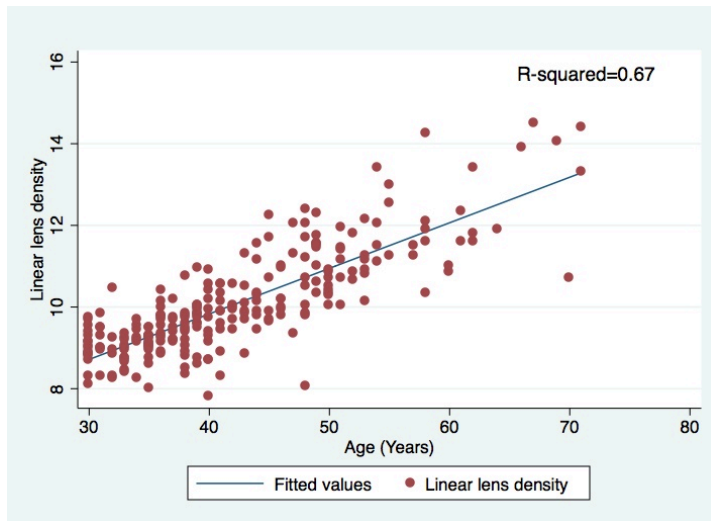
1a: Telomere length



1b: CDKN2A expression



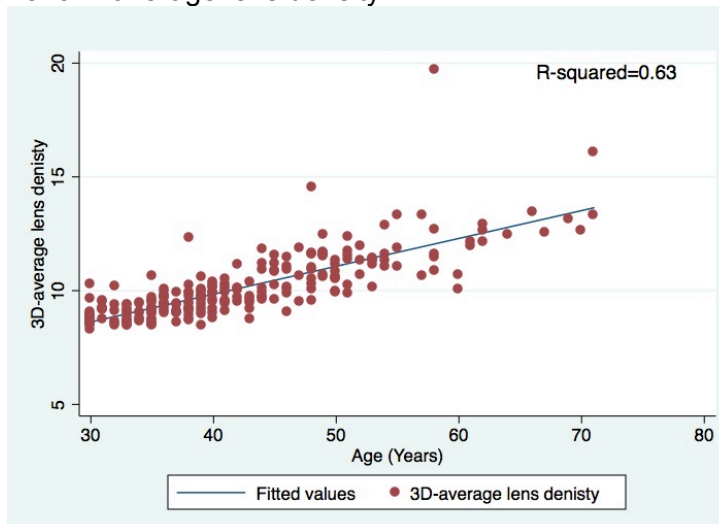
1c: Linear lens density



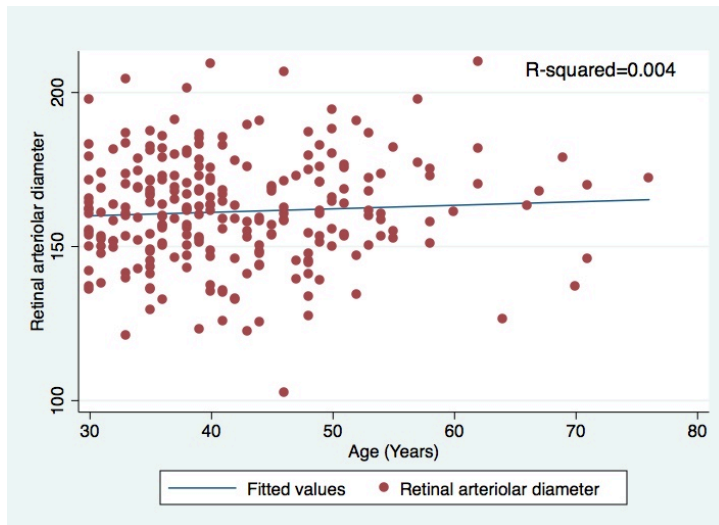
1d: Peak lens density



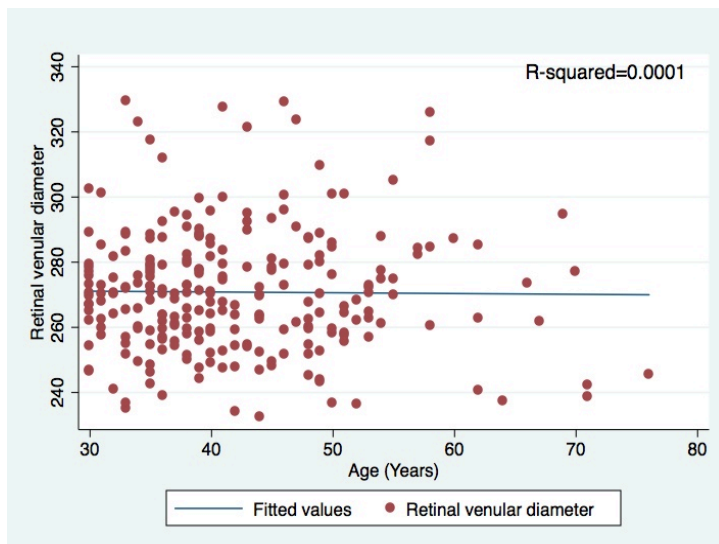
1e: 3-D average lens density



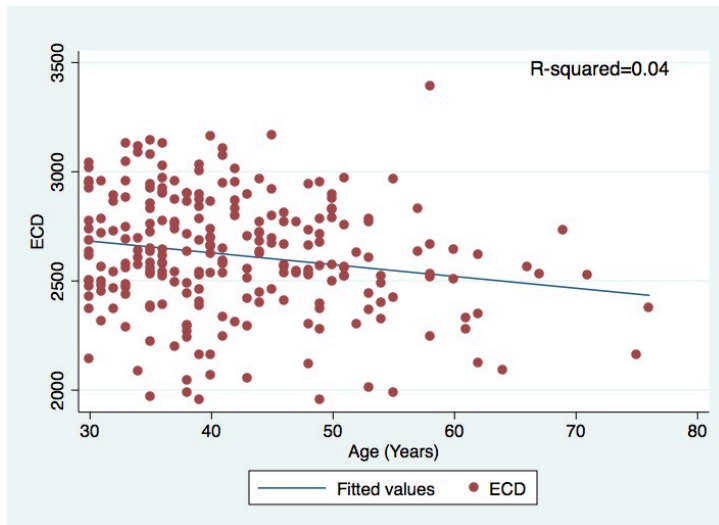
1f: Retinal arteriolar diameter



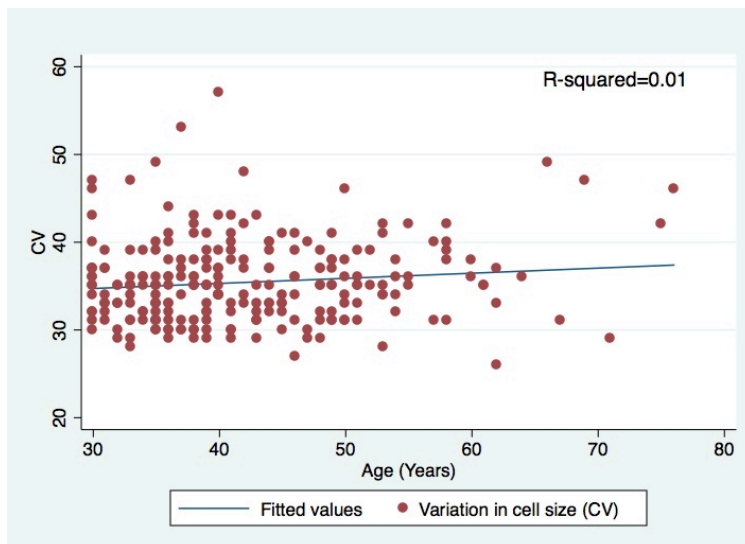
1g: Retinal venular diameter



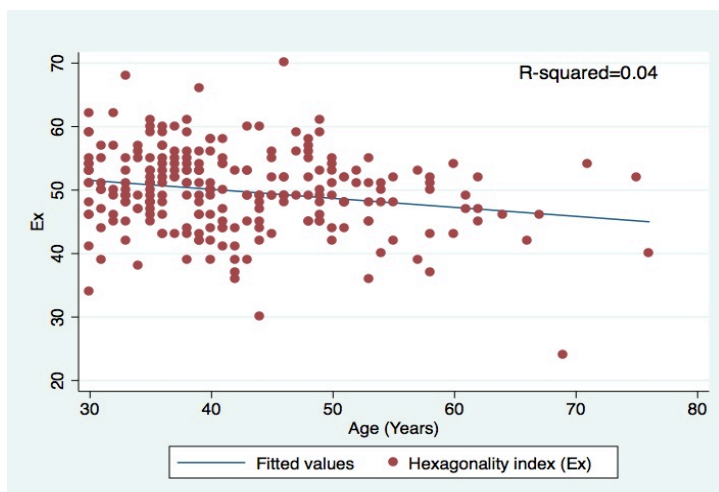
1h: Endothelial cell density



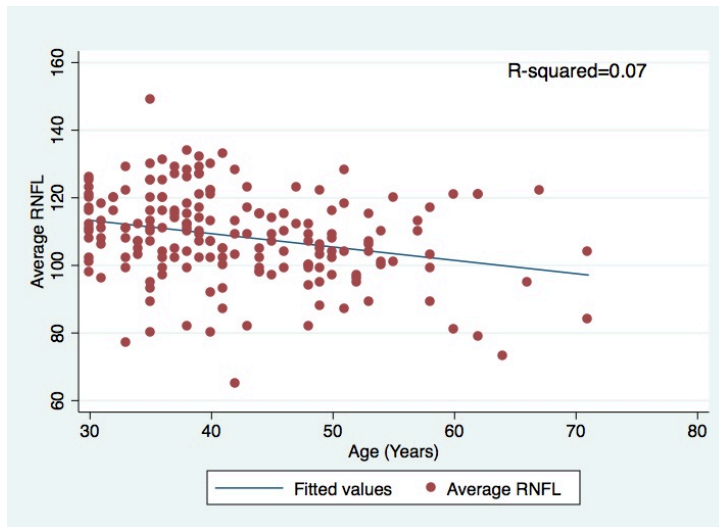
1i: Variation in cell size



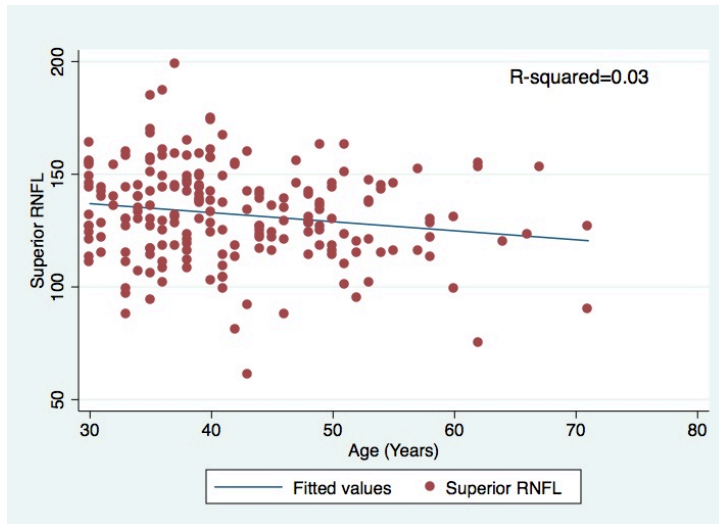
1j: Hexagonality index – proportion of cells that have 6 sides



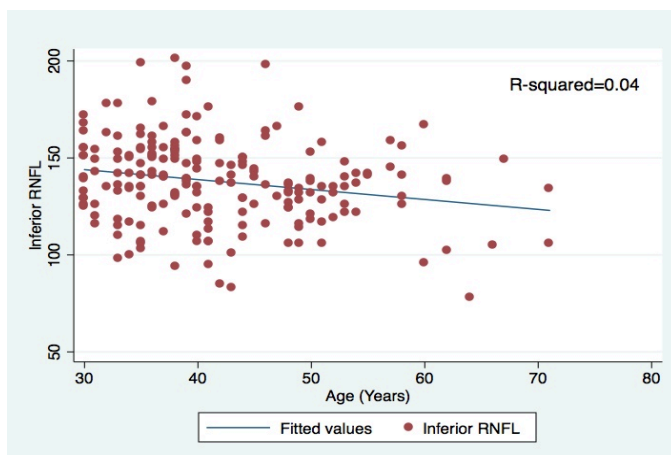
1k: Average retinal nerve fibre layer (RNFL) thickness



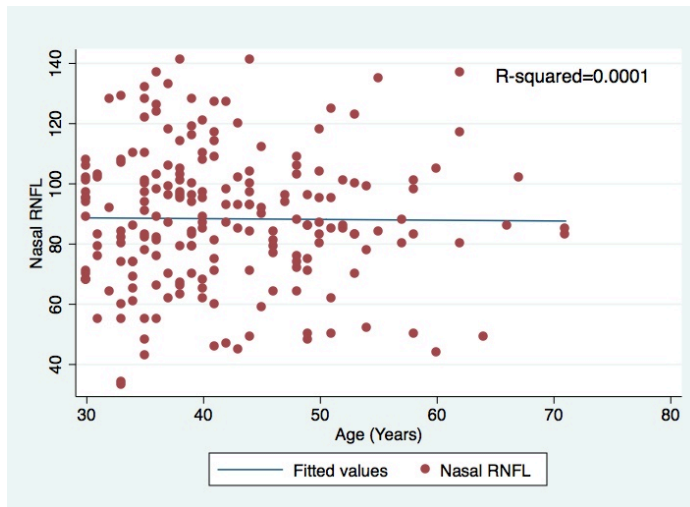
1l: Superior RNFL thickness



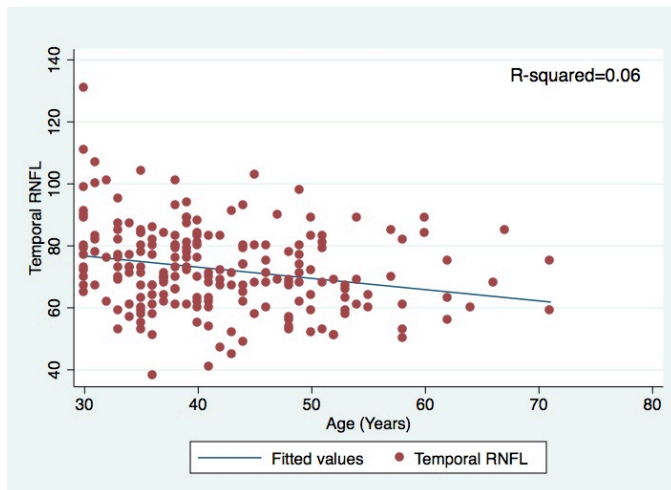
1m: Inferior RNFL thickness



1n: Nasal RNFL thickness



1o: Temporal RNFL thickness



Chapter 12

Ocular parameters of biological aging in HIV-infected individuals in South Africa: relationship with chronological age and systemic biomarkers of aging



Old Main Building, Groote Schuur Hospital and Devil's Peak in the background

Research paper investigating the association of potential ocular biomarkers of aging in HIV infection in relation to systemic biomarkers and chronological age

Cover sheet for each 'research paper' included in a research thesis

1. For a 'research paper' already published
 - 1.1. Where was the work published? **N/A**
 - 1.2. When was the work published? _____
 - 1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion **N/A**
 - 1.3. Was the work subject to academic peer review?

 - 1.4. Have you retained the copyright for the work? _____
If yes, attach evidence of retention
If no, or if the work is being included in its published format, attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published
 - 2.1. Where is the work intended to be published? **Journal of Gerontology: Biological Sciences Medical Sciences – Section A**
 - 2.2. List the paper's authors in the intended authorship order

S Pathai, HA Weiss, PG Shiels, C Gilbert, T Peto, LG Bekker, R Wood, TY Wong, SD Lawn
 - 2.3. Stage of publication – **Not yet submitted**/~~Submitted~~/~~Undergoing revision from peer reviewers' comments~~/~~In press~~

3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I designed the experiments and conducted the data collection including all ophthalmic tests. I performed DNA extraction, quantification and RNA preparation on site in Cape Town. I performed the statistical analyses with advice from Helen Weiss. Paul Shiels' laboratory team were responsible for measurement of CDKN2A levels and telomere length. I wrote the first draft of the manuscript and prepared the subsequent revisions with consideration of comments from co-authors.

Candidate's signature



Supervisor or senior author's signature to confirm role as stated in (3)



Ocular parameters of biological aging in HIV-infected individuals in South Africa: relationship with chronological age and systemic biomarkers of aging

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Keywords: Telomeres; CDKN2A; lens density; retinal vessel calibre; corneal endothelium; retinal nerve fibre layer

Running head: Ocular biomarkers of aging in HIV infection

Abstract

Background: HIV-infected individuals have increased risk of age-related morbidity despite antiretroviral treatment (ART). Several anatomic and functional ophthalmological parameters are associated with increasing chronological age. These may, therefore, potentially serve as biomarkers of aging either individually or as a composite index.

Methods: Cross-sectional study of 216 HIV-infected South African individuals receiving ART. We investigated associations between four ocular parameters (lens density, retinal vessel calibre, corneal endothelial cells and retinal nerve fibre layer thickness) and two 'cellular' biomarkers of aging (leukocyte telomere length and CDKN2A expression). We also investigated the association of these ocular parameters with frailty (a clinical correlate of aging).

Results: The median age of participants was 40 years (IQR:35-46 years) and 75% were female. The median CD4 count was 468 cells/ μ L and 94% had an undetectable viral load. All ocular parameters, telomere length and frailty were associated with chronological age, whereas CDKN2A expression was not ($R^2=0.007$, $p=0.25$). Retinal venular diameters and linear lens density values were associated with shorter telomere length (p -trend=0.04, and 0.08, respectively), whereas CDKN2A expression and frailty status were not associated with any of the ocular parameters.

Conclusions: Lens density and retinal venular calibre may be of value in the assessment of biological aging in HIV infection. Longitudinal studies are warranted to assess the integration of these data with systemic markers to develop an overall index of biological aging.

Introduction

Major reductions in HIV-associated mortality have occurred largely due to the global scale-up of antiretroviral therapy (ART). However, evidence is emerging that patients receiving ART are at an increased risk of age-related non-AIDS morbidity and mortality compared with HIV-seronegative individuals (1-3). Several of these conditions are classically associated with the normal aging process but appear to occur at an earlier age in HIV-infected persons compared to age-matched HIV-seronegative individuals. It is possible that not only are HIV cohorts aging chronologically, but they may also be undergoing accelerated biological aging.

Chronological age is an imprecise measure of biological aging, due to inter-individual differences in rates of aging. The disconnection between chronological age and lifespan has led to a search for effective and validated biomarkers of aging (BoA), defined as “biological parameters of an organism that either alone or in some multivariate composite will better predict functional capability at some late age, than will chronological age” (4). In HIV infection, heightened inflammation and immune dysfunction is likely to play a role in accelerated aging (1). This has stimulated assessment of several of biomarkers of inflammation and immune dysfunction, including C-reactive protein (CRP), interleukin-6 (IL-6) and D-dimer and their associations with age-related morbidity and HIV infection (5).

Only two validated BoA, telomere length (TL) and CDKN2A expression, have so far been found to satisfy the majority of the criteria proposed by Baker and Sprott (4). Telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes. Their DNA component shortens with somatic cell division and upon reaching a critically short length, a DNA damage signal leads to growth cycle arrest, resulting in replicative senescence (6, 7). Telomere shortening is associated with increasing chronological age and several pathologies. Telomere length (TL) may be useful as a composite measure of healthy aging, but not as a BoA when used in isolation (8, 9). Expression levels of the cell cycle regulator CDKN2A may represent a more robust BoA in healthy aging (10). CDKN2A acts as a tumour suppressor and maintains cells in a state of growth arrest, both in replicative and stress induced-senescence. Increasing levels of CDKN2A transcriptional expression occur with increasing age and decreasing function of solid organs and peripheral blood leucocytes (PBLs) (11-14). However, in disease or pro-inflammatory states such as HIV infection, evaluation of CDKN2A expression may be compromised by mechanisms such as stress-induced premature senescence (SIPS) (15). Furthermore, measurement of telomere

length may be affected by the use of nucleoside reverse transcriptase inhibitors (NRTIs) (16). Thus, novel biomarkers are required to evaluate biological aging in HIV infection (17).

Certain anatomic and functional parameters of the eye change with increasing chronological age. They may, therefore, serve as potential biomarkers of aging (18). We have reported on ocular parameters related to the accelerated aging phenotype in HIV, including retinal vessel calibre and objective measurement of lens density (19) (*and Pathai et al, 2013 JAIDS; in press*). Furthermore, we have shown frailty to be part of this phenotype (20). Frailty is a functional state characterised by an increased risk of multiple pathologies, low physical activity and slow motor performance (21). Frailty predicts cognitive and physical decline and is associated with an increased risk of morbidity and mortality, and may therefore act as a 'clinical' biomarker of aging (21). There are limited data on how these parameters correlate with cellular BoA in the context of HIV as such markers are typically used in the evaluation of healthy biological aging. In addition, there are few data relating to biological aging in sub-Saharan Africa, a region where the population of HIV-infected elderly people is rapidly expanding and where the AIDS epidemic is most severe (22).

With the need for novel BoA that could be valid in disease as well as health, the aim of this study was to investigate the association between eye parameters, frailty and cellular BoA in HIV infection.

Methods:

Study population:

Individuals aged ≥ 30 years from community HIV treatment clinic in a township of Cape Town, South Africa (Hannan Crusaid Centre, Gugulethu) were recruited as HIV-infected 'cases' as part a case-control study investigating HIV and aging (19, 20). Participants in this study were receiving ART at the time of enrolment. Socio-demographic information and medical history were obtained by interviewing participants in their first language (Xhosa or English). Data collected included factors known to affect aging (e.g. UV exposure, smoking history). All participants underwent a full ophthalmic examination including measurement of visual acuity, evaluation by slit lamp microscopy and indirect ophthalmoscopy.

The study was approved by the Ethics Committees of the London School of Hygiene and Tropical Medicine and the University of Cape Town Faculty of Health Sciences, and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Anthropometry, blood pressure and physical function including frailty assessment:

Blood pressure (BP) was measured with a digital sphygmomanometer. Mean arterial blood pressure (MABP) was defined as two-thirds of the diastolic plus one-third of the systolic BP (23). Hypertension was defined as a systolic BP of 140mmHg or higher, diastolic BP of 90mmHg or higher, or the combination of self-reported high BP diagnosis and the use of anti-hypertensive medications (24). Body mass index (BMI) was defined as weight (in kilograms)/height².

Physical frailty was defined by the presence of ≥ 3 of 5 criteria: i) unintentional weight loss (self reported and verified from clinic records where possible) ii) self-reported low physical activity, iii) self-reported exhaustion, iv) weak grip strength and v) slow walking time. Pre-frailty was defined as the presence of one or two of these criteria. Detailed information is available in the Supplementary Methods.

Blood-based biomarkers:

DNA/RNA extraction

DNA was extracted from PBLs using the Maxwell™ Automated Purification System according to manufacturer's instructions (Promega, USA). DNA concentration and purity were quantified by Nanodrop Spectrophotometer (ThermoFisher Scientific, USA). RNA was extracted using Trizol reagent (Invitrogen, UK) following manufacturer's guidelines. DNA/RNA extraction was performed in Cape Town and samples shipped on dry ice to the University of Glasgow.

Telomere length determination

TL were determined by QPCR following the method of Cawthon (25). TL determination was performed blindly using a Roche Light Cycler LC480. Briefly, telomere length analyses were performed in triplicate for each sample, using a single-copy gene amplicon primer set (acidic ribosomal phosphoprotein, 36B4) and a telomere-specific amplicon primer set (14). Refer to Supplementary Methods for further detail.

CDKN2A expression determination

Relative quantitative real-time PCR (qRT-PCR) was used to estimate mRNA levels corresponding to the candidate senescence associated gene - CDKN2A. Expression levels were measured against a reference hypoxanthine phosphoribosyltransferase (HPRT) housekeeping gene on an ABI Prism(R) 7500 Sequence Detection System. Sequences of human TaqMan™ Primer/Probe sets were designed by Primer Express algorithm (Applied Biosystems, Austin, TX, USA). The comparative threshold cycle method ($\Delta\Delta CT$)(26) was employed to quantify relative gene expression.

Ocular biomarkers

The following four ocular parameters were selected (Table 1). Detailed methods are supplied in the Supplementary Methods.

- i) Lens density: Pentacam imaging (Oculus, Wetzlar, Germany) was used to obtain “Scheimpflug images” of the lens and an objective estimate of lens density on a continuous scale. Lens density increases with increasing chronological age.
- ii) Retinal vessel calibre: Participants had stereoscopic 30° colour retinal photographs taken under pharmacological pupil dilation with a fundus camera (CF-2; Canon Inc., Tokyo, Japan). Vessel calibre indices were determined in a semi-automated manner using the IVAN computer program (Singapore Eye Research Institute, Singapore) and a standardized protocol described previously (27). Narrowing of retinal arterioles is associated with increasing chronological age (23, 28).
- iii) Corneal endothelial cell parameters: A non-contact specular microscope was used (SP02, CSO, Florence, Italy). The operator focused and aligned a real-time image of the participant’s eye. Endothelial cell parameters were automatically calculated from this image by the microscope software. Endothelial cell density (ECD) decreases with age, whereas the change in cell size (coefficient of variation) increases with age. The proportion of cells with six sides (hexagonality index) decreases with age.
- iv) Retinal Nerve Fibre Layer (RNFL): Measured using Spectral OCT/SLO optical coherence tomography (Opko/OTI Inc, Miami, FL) which uses a scanning laser diode of 830nm to provide images of ocular microstructures. A peri-papillary (around optic nerve head) protocol inbuilt in the software was used to determine the average and quadrant-specific RNFL thickness (superior, inferior, temporal and nasal). The RNFL becomes thinner with increasing age (29, 30).

Statistical analysis:

One eye was randomly selected for analysis. Where an eye was not available, for example due to trauma or corneal opacity, the contralateral eye was used. Analyses were performed using Stata 12 (Stata Corp, College Station, TX). Clinical and biological data were summarized as the median with interquartile range (IQR) or mean with standard error (SE), as appropriate. Analyses were conducted on log₁₀-transformed values of telomere length and mean CDKN2A expression to satisfy the assumption of normally distributed residuals. Results are displayed back-transformed to the original scale. Validation of the biomarkers was performed using linear regression models with age in years as a continuous or categorical variable. Ocular biomarker measurements were divided into quartiles. Univariable linear regression was performed to compare the quartiles of ocular parameters with mean telomere length and CDKN2A expression and frailty status respectively. Multivariable linear regression models were used to examine the relationships of telomere length, CDKN2A expression as the respective dependent variable with ocular biomarker quartiles and frailty status and explanatory variables (age group [30-39; 40-49; >50 years], gender, MABP; BMI, smoking, UV exposure, current and nadir CD4 count, peak and current HIV viral load) as independent variables. Marginal adjusted means for telomere length and CDKN2A expression were estimated at the mean value of covariates in the model. The Wald test was used to assess statistical significance of the association of each ocular parameter on systemic biomarker levels.

Results**Participant characteristics and biomarker distributions:**

216 participants underwent assessment. The median age was 40 years (IQR: 35-46) and 25% (n=54) were male. Characteristics of the participants by gender are given in Table S1. Women reported less alcohol consumption and cigarette use compared to men ($p < 0.0001$ for both). Men had lower BMI and lower current CD4 counts ($p < 0.0001$ and $p = 0.04$, respectively). The number of participants providing data for each parameter varied, as not every participant was able to complete all ophthalmic tests or had a blood sample available for analysis. Summary statistics for each biomarker, stratified by gender and age group are displayed in Table S2. For the majority of biomarkers, there was no evidence of gender differences; however for endothelial cell parameters, the cell density was greater in women compared to men, and women were more frail than men ($p = 0.03$ for both).

Validation of biomarkers against chronological age:

All biomarkers were initially validated against chronological age in a HIV-seronegative control group of similar age and gender, recruited as part of a case-control study (Pathai et al; unpublished data). Assessment within this study population was subsequently performed. (Table S3). Telomere length was associated with chronological age ($p=0.03$), however CDKN2A expression was not ($p=0.25$). All ocular parameters except the RNFL nasal quadrant and hexagonality index of endothelial cells were associated with chronological age, therefore these ocular parameters were not analysed further. The association of the prospective biomarkers with chronological age is presented in Supplementary Figures 1a-1o. The R-squared values of the regressions against chronological age were highest for lens density parameters (linear lens density $R^2=0.63$). All analyses thereafter were adjusted for age, gender and other possible confounding variables related to the parameter of interest.

Association of ocular parameters with blood-based biomarkers:

Shorter telomere length was associated with increasing retinal venular diameter (p -trend=0.04) - Table 2. Telomere length was not related to retinal arteriolar diameter. Table 3 reports associations with lens density: there was a trend of shorter telomere length with increasing lens density (p =trend=0.08). Endothelial cell parameters and RNFL thickness were not related to TL or CDKN2A expression (data not shown).

Association of frailty status with blood-based and ocular biomarkers:

There was no association of frailty status with either telomere length or CDKN2A expression ($p=0.54$ and $p=0.76$, respectively). Similarly, none of the ocular parameters were associated with frailty (data not shown).

Discussion

We compared several ocular parameters and frailty, a clinical correlate of aging, with TL and CDKN2A expression as established and validated biomarkers of aging. Retinal venular diameter was the most informative ocular biomarker, showing increased venular diameter with decreasing TL. Objective measurement of lens density also showed a strong trend of decreasing TL with increasing lens density. In contrast, CDKN2A expression, a validated biomarker of aging in health, was not associated with frailty or any of the ocular parameters. These findings suggest that the ocular lens and retinal vasculature, which reflect different physiological systems,

may have a role to play in the determination of biological age and aging trajectories in HIV infection, which may differ from that seen in physiological 'healthy aging'.

We have already established in this same study population that HIV infection is associated with increased frailty (20), changes in retinal vessel calibre that are consistent with aging and increased cardiovascular risk (19), and an increase in lens density in patients with severe HIV-related immunodeficiency (Pathai et al 2013; JAIDS, in press). These findings indicate the presence of an HIV-related accelerated aging phenotype, which is likely to have important clinical and health systems implications as HIV-infected populations continue to increase and live longer. Associations of these ocular and clinical parameters with validated BoA have not been investigated to date. The need for evaluated biomarkers in determining biological age is gaining increasing importance as data emerge about the excess risk of age-related morbidity in HIV-infected individuals despite suppression of viral load (17).

Of a wide range of BoA evaluated, only TL and CDKN2A are thought to meet the Baker and Sprott criterion (4). The majority of putative BoA assessed fail on the basis that they are unable to predict functional capacity in the absence of disease (31). The issue is further compounded in HIV infection as BoA are typically used to evaluate physiological, 'healthy' aging. Measurement of 'accelerated' biological aging HIV infection may involve different mechanisms, and therefore render some BoA invalid in their measurement. This is pertinent to the lack of association between CDKN2A and chronological age in our HIV-infected study population, where the disease state decouples CDKN2A expression from correlation with chronological ageing. We have found CDKN2A expression to be significantly higher in HIV-infected individuals compared to age- and gender-matched controls (Pathai et al., - unpublished data). However, as well as functioning as a tumour suppressor, CDKN2A is also a component of stress-induced premature senescence (SIPS) (15) which prevents T-cell replication following acute insult (32). The decoupling of the relationship between CDKN2A expression and chronological age in HIV infected individuals is a direct consequence of HIV-associated premature T-cell senescence (33), and lack of further T cell replication with HIV infection. SIPS causes populations of cells to 'freeze' in time with respect to age, confounding the determination of rate changes in replicative senescence and therefore biological aging. The Baker and Sprott criterion state "The biomarker should reflect some basic biological process of aging and certainly not the predisposition toward a disease

state or some error in metabolism” (4). Thus, the induction of SIPS by CDKN2A in disease states such as HIV infection suggests that this is an unreliable BoA in these circumstances.

Telomere length has also been shown to be significantly shorter in HIV-infected individuals compared to HIV-seronegative controls in this population (Pathai et al - unpublished data), and may better satisfy the Baker and Sprott criteria in disease states. However, telomere attrition is affected by psychosocial confounders, genetics and potentially by NRTIs used in the treatment of HIV infection (16), leading to the suggestion TL may be useful as a composite measure of healthy aging, but not as a BoA when used in isolation (8, 9). The trend of increasing lens density with shortening of telomere length is intuitive, and the strong association of lens density with chronological age (when compared to TL), suggests that in this population, lens density may be an attractive BoA as it is less susceptible to changes caused by systemic disease states.

The frailty phenotype was initially described in HIV-infected individuals in 2007 (34), with frailty prevalence for 55-year-old men infected with HIV for ≤ 4 years similar to that of uninfected men ≤ 65 years old. Similarly, we have found an increased prevalence of frailty in this study population (20). However, we did not detect an association between frailty and cellular BoA or ocular parameters. In contrast, in an HIV-seronegative study population from the same community we found increasing lens density to be associated with frailty status (Pathai et al., - unpublished data). This suggests that the frailty phenotype in HIV, while sharing some characteristics with the original description in the geriatric population (21), has other characteristics perhaps unique to HIV infection and/or use of ART. This is corroborated by the finding that CD4 count is also associated with the development of frailty (20, 35), suggesting that compromise of the immune system in HIV-infected individuals contributes to the development of the frailty phenotype. Furthermore, HIV-infected frail individuals have higher concentrations of pro-inflammatory cytokines such as IL-6, and tumour necrosis factor-alpha (TNF- α), and C-reactive protein (36). Age-related comorbidities and AIDS predict conversion from being non-frail to frail, and having a persistent frailty-like phenotype before HAART initiation predicts mortality (37). Collectively, these findings suggest that frailty in HIV infection is not simply an accelerated version of geriatric frailty, and so the lack of relationship with BoA at a cellular or ocular level may be expected.

Retinal arteriolar and venular calibre typically narrow with increasing chronological age (23, 28), thus shortening of TL might be expected to be associated with narrowing of venular diameter. However, our observation that shortened TL was associated with increasing retinal venular diameter is nevertheless plausible in the context of HIV infection. Inflammation is recognised as a key pathogenic process in HIV infection, and also in HIV-related accelerated aging (1). Larger retinal venular calibre is associated with systemic inflammatory markers such as CRP, fibrinogen, IL-6 and smoking, independently of age (38-40). This suggests that the heightened inflammatory processes observed in HIV infection are consistent with a biologically aged phenotype as manifest by shorter TL. Patients with HIV infection are at increased risk of developing cardiovascular disease (41). Although factors potentially contributing to this elevated risk include traditional cardiovascular risk factors and antiretroviral medications, it is likely that inflammatory and immunologic factors also contribute. Endothelial dysfunction is also associated with larger retinal venules independent of traditional cardiovascular risk factors (42). We therefore postulate that HIV-related inflammation and/or endothelial dysfunction manifests as retinal venular dilation and contributes to the biologically aged phenotype. Furthermore, retinal arteriolar narrowing which is associated with increased cardiovascular risk (43), is related to increasing duration of ART, independent of age (19). Thus, assessment of retinal vessel diameters may be a novel and non-invasive method of assessing vascular risk and biological aging in this population.

This study has some limitations. The gender composition of participants was three-quarters female, but this is reflective of the HIV epidemic in Africa. There were also differences between genders (smoking, alcohol consumption) that may truly exist or may have been misclassified (e.g. misreporting true smoking habits) and this could have confounded associations of the ocular parameters with the other biomarkers. Participants were recruited from a community of considerable socio-economic deprivation, and therefore likely to have been exposed to factors known to increase biological aging such as high UV exposure from outdoor work. Therefore, our data might over-estimate associations related to aging. Lastly, as study participants are of African ancestry, our results are generalizable to the African population.

Biomarkers are used extensively in the assessment of HIV infection, from monitoring of CD4 count and HIV RNA viral load to assess response to ART, to the use of indices such as the VACS Index to predict morbidity and mortality (44). Our

findings indicate that measurement of biological aging in HIV infection may be complicated by inflammation and immune dysfunction such that BoA validated in healthy aging are no longer accurate predictors. This suggests that other biomarkers are needed; our data indicate that measurement of lens density and retinal vessel calibre may be useful additions in the assessment of biological aging. We have proposed a research agenda to further define and validate ocular biomarkers of aging (18). Vessel calibre assessment may provide information about inflammation and systemic vascular changes, whereas lens density evaluation may be indicative of true biological aging. Longitudinal studies are warranted to assess the integration of these data with systemic markers to develop an overall estimate of biological aging and possibly mortality risk. In conclusion, the eye may prove to be a useful addition in the assessment of biological aging and to improve our understanding of HIV-related accelerated aging.

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Table 1: Biomarkers of aging, methods of measurement and the impact of aging

Anatomical site	Parameter	Method of measurement	Age-related changes
Peripheral blood leukocytes	Telomere length (TL)	qPCR	TL shortens
	CDKN2A expression	qRT-PCR to estimate mRNA levels	Increased expression
Corneal endothelium	Endothelial cell density (ECD)	Specular microscopy	Decreased ECD
	Coefficient of variation (CV)		Increased CV
	Hexagonality index (Ex)		Decreased Ex
Lens	Lens opacity	Pentacam – lens densitometry	
	Linear value		
	Peak		All increase
	3-D average		
Retina	Retinal nerve fibre layer (RNFL) thickness (Average, superior, inferior, nasal, temporal quadrants)	Optical coherence tomography (OCT)	Thinner RNFL- all quadrants
	Retinal vessel caliber		Reduced diameter
		Semi-automated retinal analysis software applied to fundus photographs	of arterioles and arterio-venous ratio (AVR)
Systemic	Frailty status		
	Non-frail (no criteria)	Assessment of walking speed,	
	Pre-frail (1-2 criteria)	grip strength, self-report of weight	Frailty status
	Frail: ≥ 3 of 5 criteria	loss, exhaustion and low physical activity	increases

Table 2: Association of retinal arteriolar and venular diameter with systemic biomarkers

Vessel parameter	TL			CDKN2A		
	N	Rel T/S	P	N	Mean	P
Retinal arteriolar quartiles (µm)						
1 st (113.16-150.13)	38	0.89 (0.81-0.97)		37	0.37 (0.29-0.47)	
2 nd (150.56-161.06)	42	0.87 (0.80-0.95)		40	0.54 (0.43-0.68)	
3 rd (161.53-171.89)	62	0.92 (0.87-0.99)		61	0.43 (0.36-0.51)	
4 th (172.79-207.80)	54	0.93 (0.87-1.00)	P-trend 0.25	53	0.44 (0.36-0.54)	0.19
Retinal venular quartiles (µm)						
1 st (218.31-258.35)	66	1.04 (0.92-1.17)		64	0.54 (0.39-0.74)	
2 nd (258.78-269.99)	57	0.89 (0.83-0.95)		56	0.41 (0.34-0.49)	
3 rd (270.24-281.54)	40	0.87 (0.79-0.97)		40	0.48 (0.36-0.63)	
4 th (281.7-326.08)	33	0.74 (0.63-0.86)	P-trend 0.04	31	0.31 (0.20-0.51)	0.15

Adjusted for age, gender, smoking, BMI, hypertension and venular/arteriolar retinal calibre (where appropriate) and HIV-related co-variates (current and nadir CD4 count, current and peak viral load, ART duration and ART type)

Table 3: Association of lens density with systemic biomarkers, n=198

Lens density**	TL			CDKN2A		
	N	Rel T/S	P	N	Mean	P
Linear quartiles						
1 st (7.5-9.2)	48	0.95 (0.87-1.03)		47	0.42 (0.33-0.55)	
2 nd (9.25-9.8)	54	0.97 (0.90-1.04)		53	0.45 (0.37-0.55)	
3 rd (9.85-10.7)	48	0.87 (0.81-0.94)		48	0.44 (0.36-0.55)	
4 th (10.8-13.9)	48	0.84 (0.77-0.93)	P-trend 0.08	46	0.48 (0.36-0.62)	0.94

Adjusted for age, gender, smoking, UV exposure and HIV-related parameters (current and nadir CD4 count, current and peak viral load, ART duration and ART type)

** - Measured on a continuous scale 0-100, 100 being an opaque (completely dense) lens
4th quartile denotes aged phenotype

Supplementary Methods

Telomere length determination:

Quality control parameters for the amplifications comprised a cut off of 0.15 for the standard deviation (SD) of the threshold cycle (Ct) for sample replicates. At a SD above 0.15 the sample was reanalysed. The average SD across plates was 0.05. Relative telomere length was estimated from Ct scores using the comparative Ct method after confirming that telomere and control gene assays yielded similar amplification efficiencies. This method determines the ratio of telomere repeat copy number to single copy gene number (T/S) ratio in experimental samples relative to a control sample DNA. This normalised T/S ratio was used as the estimate of relative telomere length (Relative T/S). The inter-assay variation was assessed by comparing the relative telomere estimates (T/S ratio) estimates across assays for the positive controls, assayed on every assay plate. The average inter-assay coefficient of variance was 0.6% for telomere length and 0.23% for 36B4.

Frailty determination

All of these five components described in the original phenotype by Fried et al [1] were used to determine the presence of frailty. However, we used the proxy described by Önen et al [2] for the physical activity measure (see Table 1 below for description). Grip strength of the dominant hand was measured three times using a grip dynamometer (Jamar Plus+ Digital Hand Dynamometer, Jamar, US). The average of three weight measurements was recorded in kilograms (kg) to one decimal point. Walking time was assessed using the method of Cesari et al [3]. The average of two trials (in m/s) was used for analysis. Participants were excluded from the determination of grip strength if they had pain or arthritis of the dominant hand, and excluded from the walking test if they had paralysis of an extremity or side of the body, or needed to use a walking aid.

Lens density measurement:

Following pupil dilatation with 1% tropicamide, two lens density measurements were made on each eye, and the mean value calculated. The instrument automatically calculated the quality and reliability of a captured image. If an image was found to be of poor quality (i.e. not 'OK' on the image quality specification), the measurement was repeated. Reproducibility of the lens density evaluation in two scans was performed for 50 eyes. Lens densitometry output values were extracted from the image captures in a masked fashion. Image section 90-270 degrees was used for the right eye and image section 270-90 degrees was used for the left eye [4]. All of the densitometry metrics available from the scan (linear, peak and 3D) were analysed as they reflect different parameters of the lens. The lens density output was presented on a continuous scale from 0 (transparent) to 100 (fully opaque).

Retinal vessel measurement

All participants had stereoscopic 30 degree colour retinal photographs taken of both eyes under pharmacological pupil dilation with a fundus camera (model CF-2; Canon Inc., Tokyo, Japan). Images were centred on the optic disc. Vessel calibre indices were determined in a semi-automated manner using the IVAN computer program (Singapore Eye Research Institute, Singapore) using a standardized protocol described previously [5]. In summary, the 6 largest arterioles and venules in a ring-shaped area located between 0.5 and 1.0 disc diameter from the optic disc margin were identified (Figure 1). Computer software measured the calibre of these individual vessels, then combined them into 2 summary variables for the eye: the projected calibre size of the central retinal artery (central retinal artery equivalent [CRAE]), and the projected calibre size of the central retinal vein (central retinal vein equivalent [CRVE]), using formulas derived by Parr and Spears [6,7] and Hubbard [8], with revision by Knudtson [9]. A retinal photograph was considered ungradable if eyes had <4 acceptable measurements of either vessel type. The intergrader and intragrader grading reliabilities were assessed using a random subsample of 100 photographs reviewed four weeks after the initial grading. The intra- and intergrader intraclass correlation coefficients ranged from 0.71 to 0.93. Retinal arteriolar and venular calibre are highly correlated, and to account for potential confounding we adjusted for the fellow vessel in multivariable analyses (i.e. adjustment for arteriolar calibre in analyses of venular calibre and vice versa) [10].

Table 1: Frailty criteria: adapted from Fried et al.[1] and Önen et al.[2]

Criteria	Definition			
Unintentional weight loss	>10 pounds weight loss documented in last year or ≥5% of previous year's body weight			
Low physical activity*	Participants answering 3 when asked whether their health limits vigorous activities such as running, lifting heavy objects 1= not at all, 2 = yes, limited a little or 3 = yes, limited a lot			
Exhaustion	Participants answering 2 or 3 to either one of two statements – “How often have you felt that:” a) Everything you did was an effort or b) I could not ‘get going’ 0 = rarely (<1 day), 1= some of the time (1-2 days), 2 = occasionally (3-4 days) or 3 = most of the time (5-7 days)			
Weak grip strength	Male BMI kg/m ²	Kg	Female BMI kg/m ²	Kg
	≤24	≤29	≤23	≤17
	24.1-26.0	≤30	23.1-26.0	≤17.3
	26.1-28.0	≤30	26.1-29	≤18
	>28	≤32	>29.0	≤21
Slow walking time	Male height (cm)	Seconds	Female height (cm)	Seconds
	≤173			
	>173	≥7	≤159	≥7
		≥6	>159	≥6

*Estimation of physical activity adopted from Önen et al.[2]; the estimation of physical activity described in the original phenotype used a weighted score of kilocalories expended

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Table S1: Characteristics of study participants, n=216

Variable	Male (N=54) N (%)	Female (N=162) N (%)	P
Age (mean ± SE)	41.5±0.9	41.2±0.6	0.82
Median age, IQR	40 (37-46)	40 (35-47)	0.52
Age, years by group			
30-39	25 (46.3)	80 (49.4)	
40-49	22 (40.7)	52 (30.1)	
>50	7 (13.0)	30 (18.5)	0.43
Education			
Did not complete high school	9 (16.7)	16 (9.9)	
Completed high school	45 (83.3)	146 (90.1)	0.18
Income			
<ZAR1000/month	32 (59.3)	89 (54.9)	
>ZAR1000/month	22 (40.7)	73 (45.1)	0.58
Location of work			
Outdoors or grant-holder	38 (70.4)	98(60.5)	
Indoors	16 (29.6)	64(39.5)	0.19
Housing			
Formal	24 (44.4)	88 (54.3)	
Informal	30 (55.6)	74 (45.7)	0.21
Smoking status			
Non-smoker	32 (59.3)	152 (93.8)	
Smoker; <10 years	5 (9.3)	5 (3.1)	
Smoker >10 years	17(31.4)	5 (3.1)	<0.0001
Alcohol			
Nil	25 (46.3)	126 (77.8)	
<1L/week	18 (33.3)	23 (14.2)	
>1L/week	11 (20.4)	13 (8.0)	<0.0001
Hypertension			
No	42 (77.8)	111 (68.5)	
Yes	12 (22.2)	51 (31.5)	0.20
BMI			
<20	12 (22.2)	5 (3.1)	
20-24.9	29 (53.7)	31(19.1)	
25.0-29.9	10 (18.5)	56 (34.6)	
>30	3 (5.6)	70 (42.2)	<0.0001
Tuberculosis status			
No history	8 (14.8)	52 (32.1)	
Previous history	43 (79.6)	109 (67.3)	
Current	3 (5.6)	1 (0.6)	0.005
Current CD4 count, median (IQR)	410 (270-610)	481 (345-607)	0.04
Nadir CD4 count, median (IQR)	135 (81-166)	122 (70-174)	0.69
Viral load			
<50 copies/ml	49 (90.7)	154 (95.0)	
>50 copies/ml	5 (9.3)	8 (5.0)	0.25
Duration of ART*, median (IQR)	55 (41-72)	60 (33-76)	0.96

*ART; anti-retroviral treatment

Table S2: Median values of candidate biomarkers by gender and age group
P-value for difference between genders

Measure	Age group, years	N	Men		N	Women		P-value
			Median	IQR		Median	IQR	
Systemic biomarkers								
Telomere length Rel T/S	Overall	48	0.96	0.78-1.09	156	0.92	0.78-0.92	0.51
	30-39	23	1.00	0.80-1.13	78	0.97	0.79-1.10	
	40-49	20	0.94	0.81-1.07	49	0.91	0.78-1.11	
	>50	5	0.74	0.64-1.27	29	0.82	0.74-1.00	
CDKN2A expression	Overall	47	0.46	0.24-0.72	152	0.50	0.29-0.70	0.48
	30-39	22	0.34	0.21-0.65	77	0.50	0.27-0.70	
	40-49	20	0.50	0.25-0.81	46	0.47	0.31-0.66	
	>50	5	0.68	0.41-0.81	29	0.56	0.42-0.74	
Lens density Scale 0-100								
Linear	Overall	52	9.8	9.2-10.5	157	9.8	9.3-10.5	0.42
	30-39	24	9.2	8.9-9.7	77	9.4	9.0-9.6	
	40-49	22	10	9.8-10.6	50	10.3	9.8-10.9	
	>50	6	11.0	10.5-12.1	30	11.5	11.0-12.0	
Peak	Overall	52	17.9	14.9-19.5	157	17.9	15.6-20.1	0.57
	30-39	24	18.0	15.4-18.8	77	16	14.7-18.1	
	40-49	22	16.8	14.8-19.1	50	18.5	16.1-21.1	
	>50	6	21.6	15.8-25.0	30	20.5	18.8-24.7	
3-D average	Overall	52	9.7	9.1-10.3	157	9.8	9.1-10.8	0.33
	30-39	24	9.1	8.8-9.7	77	9.1	8.9-9.5	
	40-49	22	10	9.5-10.3	50	10.3	9.8-10.8	
	>50	6	11.2	10.5-12.5	30	11.7	11.0-12.4	
Retinal vessel calibre μm								
CRAE	Overall	50	161.7	151.5-170.6	157	164.8	152.5-173.4	0.36
	30-39	22	160.7	151.5-167.6	77	167.1	155.5-176.5	
	40-49	22	164.1	158.7-172.4	51	161.7	152.3-171.9	
	>50	6	139.3	122.1-170.3	29	162.4	144.8-169.4	
CRVE	Overall	50	262.2	250.6-273.9	157	266.2	255.7-279.9	0.15
	30-39	22	263.8	258.3-274.4	77	269.9	259.8-282.8	
	40-49	22	260.9	246.2-272.9	51	263.3	251.9-276.1	
	>50	6	260.7	238.1-277.4	29	263.7	254.2-271.3	
Endothelial cell parameters								
ECD	Overall	48	2598	2337-2730	150	2690	2463-2858	0.03

	30-39	23	2671	2514-2850	74	2720	2554-2880	
	40-49	20	2466	2125-2762	49	2665	2400-2816	
	>50	5	2402	2122-2590	27	2566	2382-2729	
CV	Overall	48	36	34-38	150	36	33-39	0.84
	30-39	23	36	34-39	74	35	32-37	
	40-49	20	36	33-38	49	37	33-39	
	>50	5	36	33-41	27	38	34-41	
Ex	Overall	48	49	45-54	50	50	45-54	0.91
	30-39	23	49	44-54	74	50	45-53	
	40-49	20	50	46-54	49	50	46-55	
	>50	5	46	38-53	27	48	42-51	
RNFL thickness, μm								
Average	Overall	45	110	101-116	143	112	102-118	0.47
	30-39	21	115	106-117	74	114	107-122	
	40-49	19	107	98-115	42	114	100-117	
	>50	5	102	93-113	27	102	89-113	
Superior	Overall	50	132	124-145	147	137	121-151	0.22
	30-39	23	133	125-145	74	142	128-157	
	40-49	21	132	125-145	46	131	120-146	
	>50	6	119	100-130	27	126	109-142	
Inferior	Overall	50	133	121-144	149	141	124-152	0.18
	30-39	23	141	126-156	75	144	129-154	
	40-49	21	129	123-142	46	142	118-149	
	>50	6	119	96-134	28	124	110-141	
Nasal	Overall	50	92	76-104	147	94	76-103	0.92
	30-39	23	89	76-103	74	93	78-101	
	40-49	21	94	77-107	46	94	75-110	
	>50	6	87	52-134	27	91	61-99	
Temporal	Overall	50	73	64-81	148	73	63-84	0.63
	30-39	23	74	65-83	74	78	67-88	
	40-49	21	71	64-77	46	69	57-78	
	>50	6	65	49-82	28	66	60-73	
Frailty status		N	%		N	%		P-value
Non-frail	Overall	20	37.0		50	30.8		0.56
	30-39	10	40.0		32	40.0		
	40-49	9	40.9		16	30.8		
	>50	1	14.3		2	6.7		
Pre-frail	Overall	27	50.0		80	49.4		0.68
	30-39	12	48.0		38	47.5		
	40-49	9	40.9		30	57.7		

	>50	6	85.7		12	40.0	
Frail	Overall	7	13.0		32	19.8	0.03
	30-39	3	12.0		10	12.5	
	40-49	4	18.2		6	11.5	
	>50	0	0		16	53.3	

Table S3: Regression coefficients of biomarkers with chronological age in years

Biomarker	N	Coefficient	R-squared	P-value
Telomere length	204	-0.002	0.02	0.03
CDKN2A	199	0.003	0.007	0.25
<i>Lens density</i>				
Linear	209	0.11	0.63	<0.0001
Peak	209	0.23	0.18	<0.0001
Average 3D	246	0.13	0.71	<0.0001
<i>Retinal vessel calibre</i>				
Arteriolar diameter	207	-0.40	0.03	0.01
Venular diameter	207	-0.58	0.06	0.001
<i>Endothelial cell parameters**</i>				
ECD	198	-9.91	0.06	<0.0001
CV	198	0.13	0.06	<0.0001
Ex	198	-0.12	0.01	0.12
<i>Retinal nerve fibre layer thickness</i>				
Average	188	-0.60	0.14	<0.0001
Superior	197	-0.89	0.10	<0.0001
Inferior	199	-0.95	0.11	<0.0001
Nasal	197	-0.20	0.005	0.30
Temporal	198	-0.46	0.06	<0.0001

Linear regression models using age in years as a continuous variable in years

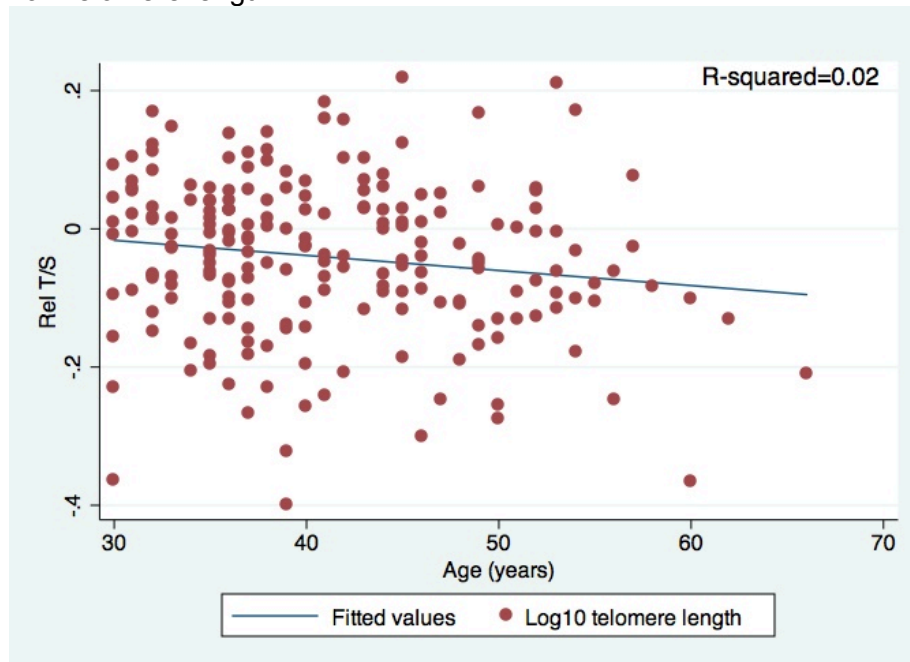
**ECD: endothelial cell density - lowest quartile denotes aged phenotype

CV: coefficient of variation – i.e. difference in cell shape; highest quartile denotes aged phenotype

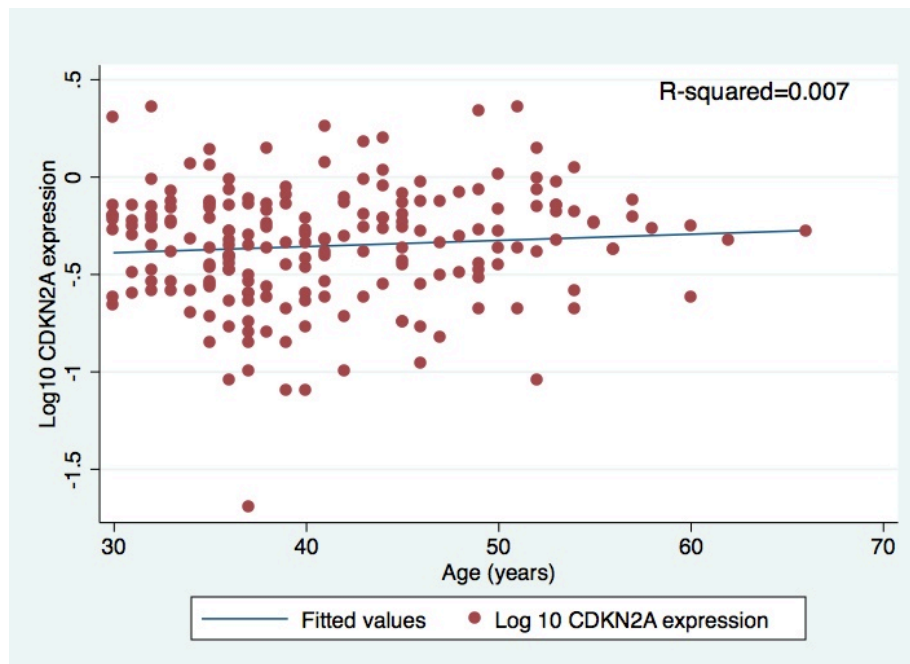
Ex: Hexagonality index – i.e. proportion of cells that are hexagonal; lowest quartile denotes aged phenotype

Supplementary Figures – Scatter plots of biomarkers – linear regression with chronological age

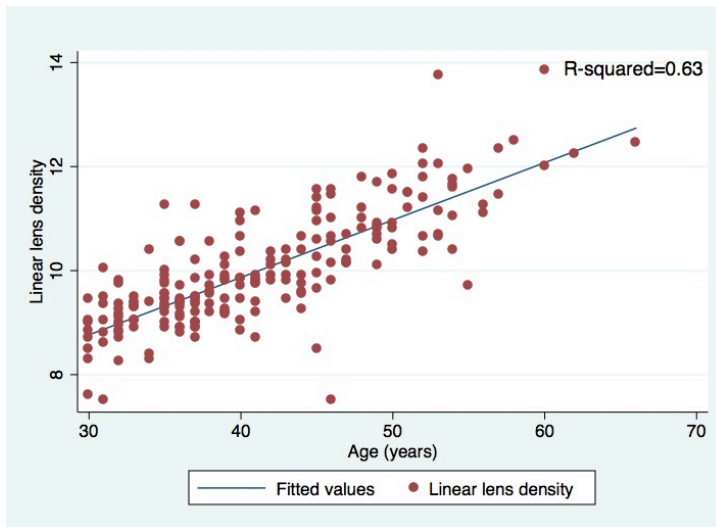
1a: Telomere length



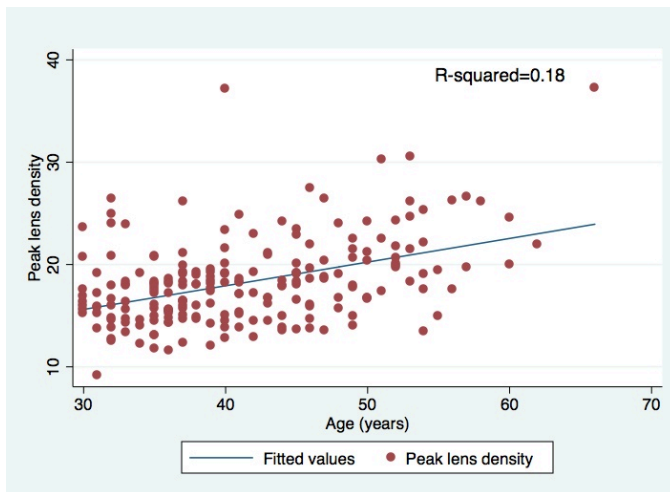
1b: CDKN2A expression



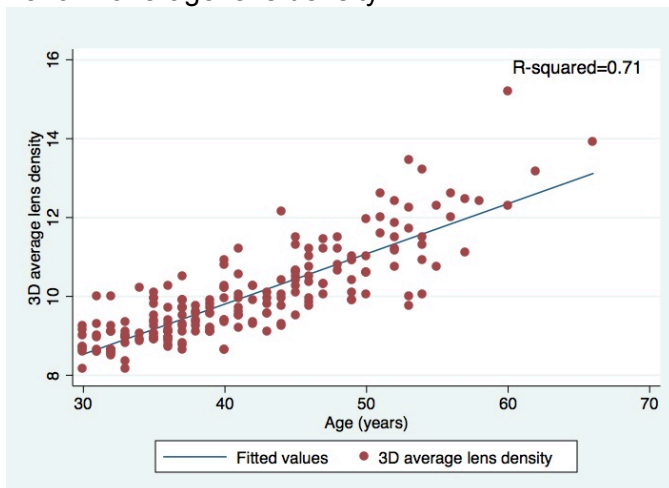
1c: Linear lens density



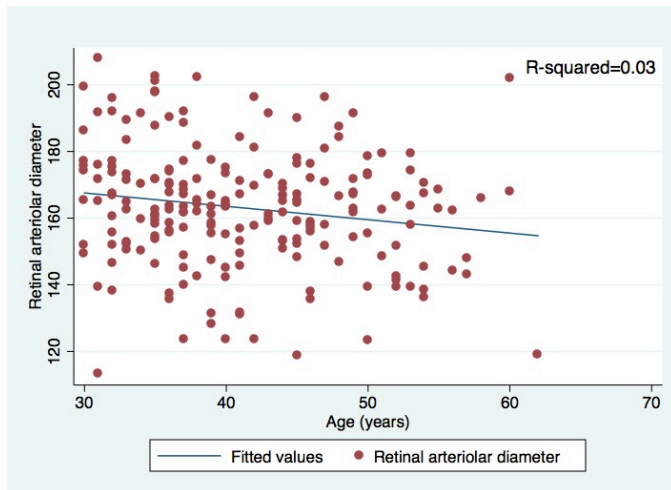
1d: Peak lens density



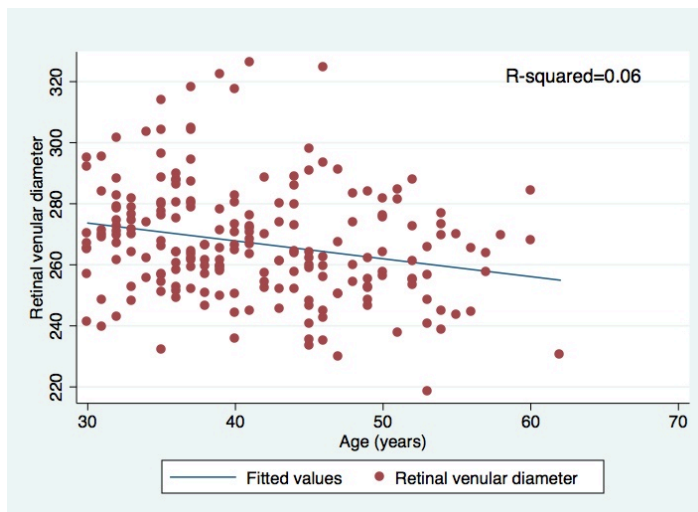
1e: 3-D average lens density



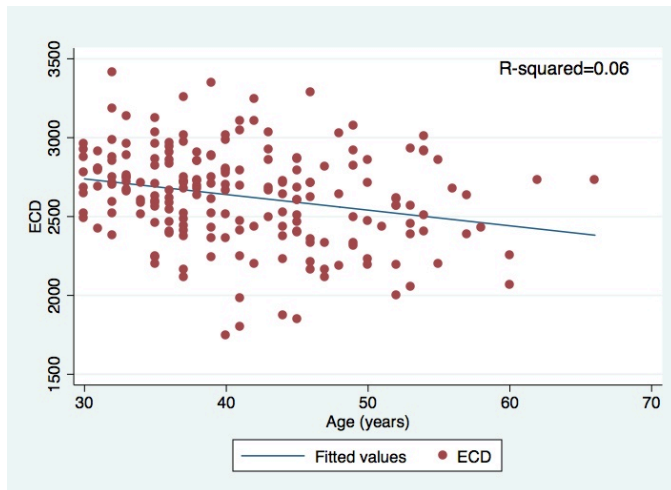
1f: Retinal arteriolar diameter



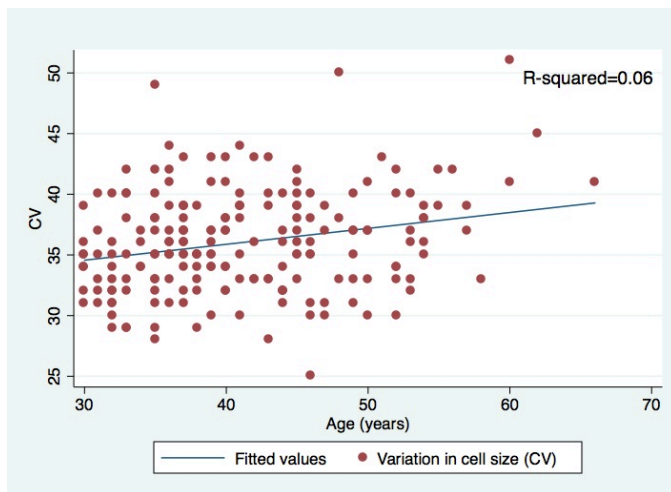
1g: Retinal venular diameter



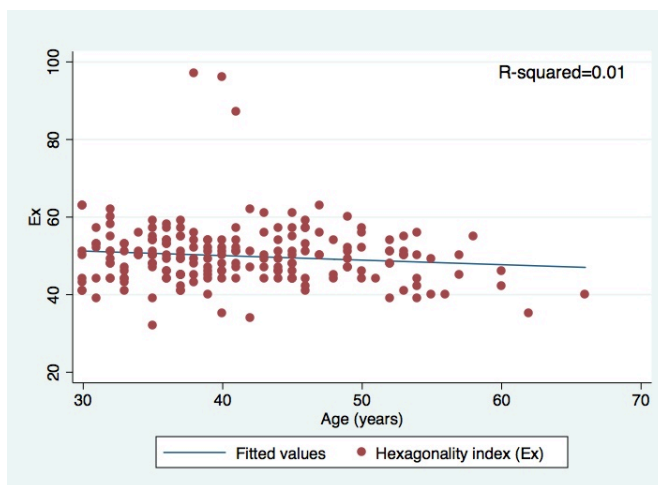
1h: Endothelial cell density



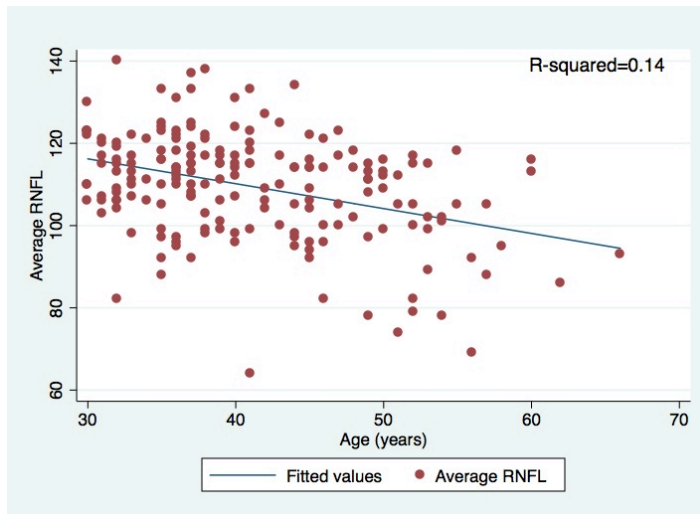
1i: Variation in cell size



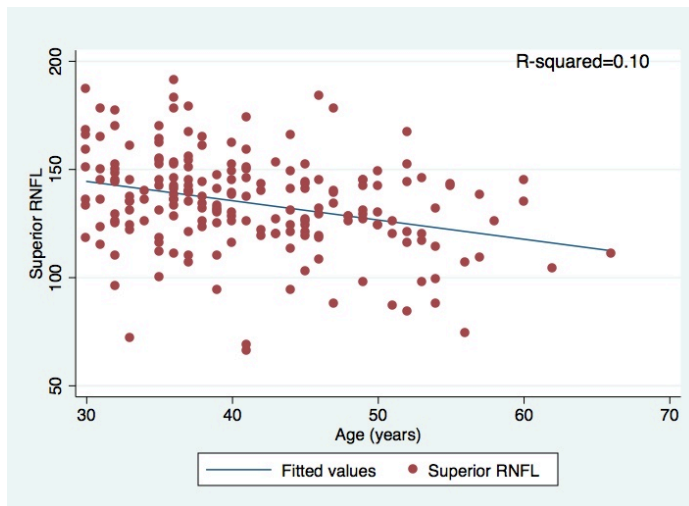
1j: Hexagonality index – proportion of cells that have 6 sides



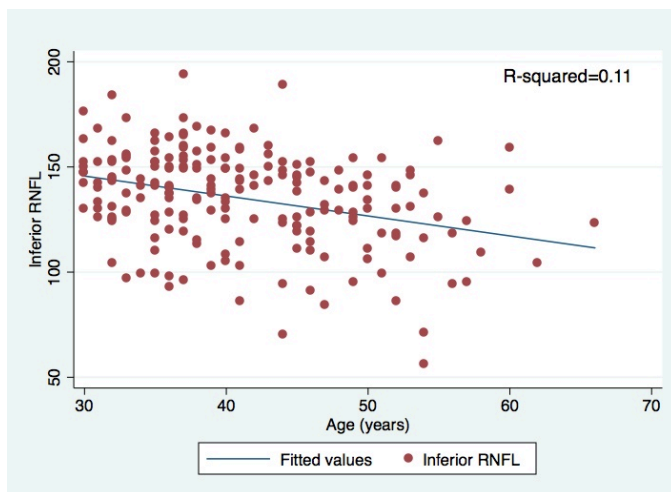
1k: Average retinal nerve fibre layer (RNFL) thickness



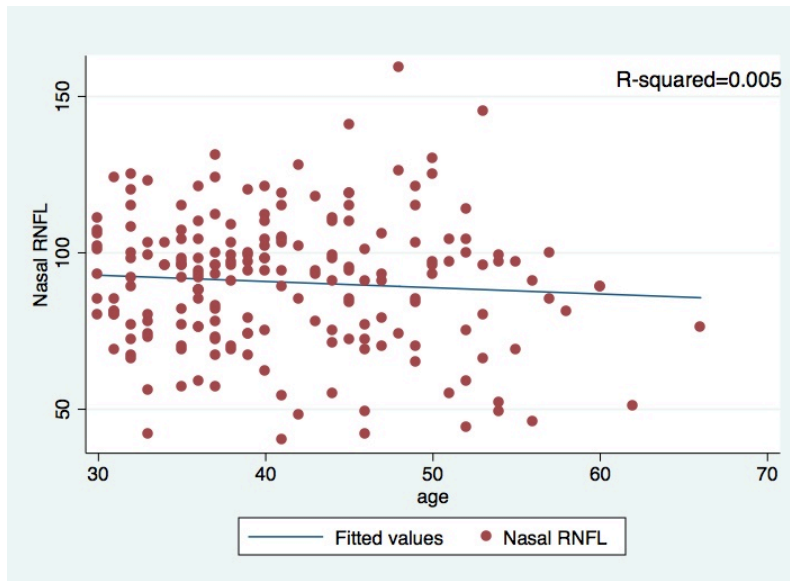
1l: Superior RNFL thickness



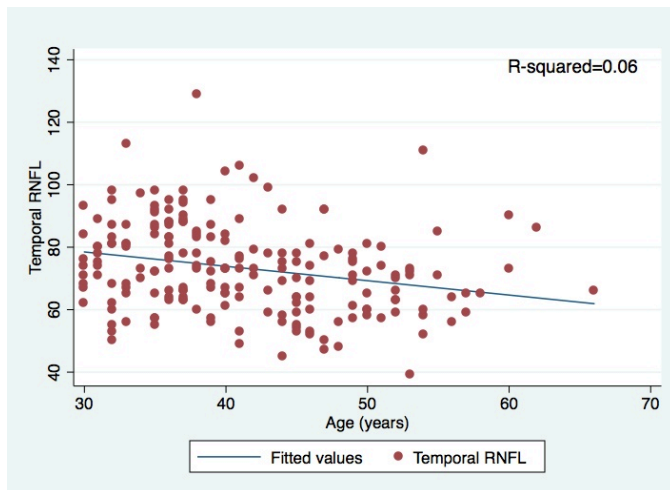
1m: Inferior RNFL thickness



1n: Nasal RNFL thickness



1o: Temporal RNFL thickness



Chapter 13

Overall conclusions and future work



Table Mountain, Cape Town

General discussion drawn from all of the contributing work including details of ongoing projects and potential future studies

13.1 Summary of findings

The proportion of individuals aging with HIV infection continues to increase worldwide [1]. The aim of this study was to assess whether HIV contributes to accelerated aging in a sub-Saharan African population; a region where there are few data relating to this concept. The eye was used as a model of aging as several of its component structures can be objectively measured and assessed. Furthermore, there are limited data on the eye and visual morbidity in HIV-related accelerated aging.

This study demonstrated accelerated biological aging, as measured by telomere length determination and CDKN2A expression, in HIV-infected individuals compared to age- and gender- frequency-matched controls. The observation of accelerated biological aging was corroborated by the finding that HIV is associated with clinical frailty, and that current CD4 count is an independent predictor of frailty status. Within the eye, increasing duration of ART was independently associated with both narrower retinal arterioles and thinning of the RNFL; both of these changes are also associated with increasing chronological age in 'physiological' aging. Greater lens density was observed in participants on ART with low nadir CD4 counts suggesting that higher levels of immunodeficiency contribute to the development of the accelerated aging phenotype. Investigation of the corneal endothelium, which provides a unique view of a cellular monolayer, demonstrated that HIV infection was associated with greater variation in cell size, a feature of cellular senescence. In HIV-infected individuals, low endothelial cell density, another feature of cellular aging, was associated with current CD4 count. These data suggest that as well as increased biological aging at a cellular and systemic level, ocular aging also occurs as part of the accelerated aging phenotype in HIV infection, particularly in clinical sub-groups such as those with greater levels of immunodeficiency pre-ART or active viremia despite ART.

Table 13 summarizes the main findings and outcomes from this study.

Table 13: Main findings from studies in this population

Parameter	Method of measurement	Sample size	Outcome
Telomere length CDKN2A expression	qPCR from PBL	486 236 HIV+/250 HIV-	Telomere length shorter in HIV+ group CDKN2A expression higher in HIV+ group Participants on ART with undetectable VL – shorter telomere length and greater CDKN2A expression associated with lower CD4 counts
Frailty	Clinical assessment	504 248 HIV+/256 HIV-	HIV associated with frailty – OR: 2.14 (1.16-3.92), p=0.01 Low current CD4 count (<500cells/ μ L) independently associated with frailty – OR: 2.84 (1.02-7.92), p=0.04
Retinal vessel calibre	Semi-automated analysis of retinal vessel diameter	491 242 HIV+/249 HIV-	Similar retinal arteriolar and venular diameters between cases and controls. Retinal arteriolar diameters narrowed with increasing duration of ART, independently of age, and with HIV VL >10,000 copies/mL while on ART

Lens density	Objective measurement using Pentacam	490 242 HIV+/248 HIV-	Adjusted lens densities similar by HIV serostatus Nadir CD4 count <200 cells/ μ L associated with higher lens density (in participants on ART) Lens density weakly associated with HIV viremia despite ART
Corneal endothelium	Specular microscopy to obtain ECD, variation in cell size and hexagonality index	491 242 HIV+/249 HIV-	HIV infection associated with increased variation in cell size – OR: 1.67 (1.00-2.78), $p=0.04$ Among HIV group, low ECD associated with current CD4 count <200 cells/ μ L – OR: 2.77 (1.12-6.81), $p=0.03$
Retinal nerve fibre layer	Spectral optical coherence tomography	428 225 HIV+/203 HIV-	CS score lower in HIV+ group compared to HIV-
Contrast sensitivity	Pelli-Robson chart		Predictors of low CS in HIV+ group: frailty and HIV VL >2 log copies/mL Longer ART duration associated with decreased thickness of inferior and nasal RNFL quadrants Superior RNFL thickness greatest in ART-naïve participants

13.2 Limitations of the study

Knowledge of time since exposure to HIV (date of HIV sero-conversion) would have been ideal. In practice this is hard to define. The nature of the infection means the majority of individuals are unaware when they have sero-converted, and so ascertainment of the time of infection is impossible. Large sero-converter cohorts do exist - CASCADE is a collaboration of 23 cohorts: 20 cohorts from Europe, two from Australia, and one from Canada [2]. In the CASCADE cohort the date of HIV sero-conversion is estimated as the midpoint between the first positive HIV antibody test and the last documented negative HIV antibody test result. This type of information is not available for this study population, or indeed for many other cohorts. Other investigators have used surrogate markers of exposure such as time since nadir CD4 count (pre-ART), or a minimum duration of ART. In this study in Cape Town the clinic database was well-maintained [3]; and the majority of participants had complete data for HIV-related parameters.

A cross-sectional study design means that the temporal relationship between exposures and outcome cannot be definitively established. In addition, the small proportion of ART-naïve participants enumerated means that inferences about the role or contribution of ART cannot be made. Low recruitment of these participants may have been for two reasons: firstly, the majority of participants newly diagnosed with HIV and fulfilling the criteria for ART in South Africa were under the age of 30, thus recruitment of participants fulfilling the study entry criteria had to use a smaller pool of individuals. Secondly, ART-naïve patients, by the nature of their clinical status tended to be in poor health and were not willing to travel in order to attend for a full assessment at the study site. Ultimately, it would be ideal to collect longitudinal data, and this current study may be used to inform future related work (See Section 13.5).

All study participants are subject to some selection biases. In the Gugulethu HIV clinic where the HIV-infected individuals were enumerated approximately 8-10% of individuals die in the first year of CART, and 1-2% per year thereafter [3,4]. Those individuals with more advanced HIV (and likely greater measures of aging) may have been at greater risk of mortality. In addition, it is possible that clinic attendees with eye symptoms were more likely to agree to participate in the study. However, previous experience when collecting initial

pilot data suggested that all patients from the Gugulethu clinic were keen to avail of an ophthalmic examination regardless of symptoms.

Measurement of other systemic biomarkers would have been ideal, particularly those that have already been demonstrated to be raised in HIV infection in other studies e.g. D-dimer, CRP, soluble CD14 [5,6]. Budgetary and time constraints precluded the investigation of these markers. However, serum has been saved from the majority of participants and could be used for future work (see below). Other ophthalmic tests e.g. visual fields, extensive colour vision testing could have been performed to add to the body of ocular parameters measured, however the additional time to perform these tests (with limited study personnel) would have reduced the number of individuals able to participate in the study, and thus the sample size. Logistics and practical issues limited the total sample size initially determined from power calculations. However, the sample size obtained for the study still provided adequate power to detect the required differences between the two groups (Chapter 4, section 4.3).

13.3 Does HIV accelerate the aging process?

The finding of shortened telomere length and increased CDKN2A expression in HIV-infected individuals compared to age- and gender-matched HIV-seronegative controls provides important evidence that HIV infection is associated with increased biological aging in this study population. The study design does not permit assessment of a temporal relationship or any inferences relating to causation. However, the finding of increased frailty in the HIV-infected population adds to the body of evidence of increased biological aging in HIV. Recruitment of the study populations from communities with similar socio-economic deprivation should have minimized the possibility of confounding due to differential risk behaviours and exposures between the two populations. At cellular (CDKN2A, telomere length) and systemic (frailty) levels, HIV-infected individuals in this study appeared to display greater biological aging compared to their uninfected counterparts.

The assessment of accelerated aging using ocular parameters is more complex to understand. The studies indicate that there was little difference in measurement of ocular parameters between the two groups overall, for example lens density and retinal vessel measurements were similar between HIV-infected and HIV-seronegative individuals. The

main differences were found in sub-groups such as those with greater levels of immunodeficiency or HIV viremia despite ART. A plausible explanation is that ocular changes related to aging may take place over a longer period of time. It is possible that the aging trajectory in this South African population is different compared to US and European HIV populations, as it is likely that African HIV populations will have experienced a shorter duration of ART and initiated treatment at lower CD4 counts (Figure 3b, Chapter 3). This may lead to differences in manifestations of ocular aging. Longitudinal studies in different geographical populations would be needed to assess this definitively. It is also unclear whether HIV infection or treatment with ART is primarily responsible for ocular aging. In addition, epigenetic and genetic variation may contribute to an individual's susceptibility to non-HIV age-related morbidity including ocular structures and their function.

13.4 Does the eye represent a 'good' model of aging?

Validation of the ocular parameters against chronological age in HIV-seronegative individuals indicated that all ocular parameters were associated with chronological age (Chapter 11). Indeed, the R-squared values in linear regression models to assess the relationship between the prospective biomarkers and chronological age were considerably higher for the majority of ocular parameters when compared to telomere length and CDKN2A expression. Thus, several of the structural and functional parameters of the eye may have use as biomarkers of aging. Longitudinal data will be necessary to definitively assess this. Of note, objective measurement of lens density was strongly associated with chronological age and with CDKN2A expression in healthy controls. The ocular lens may be particularly well-suited to a role as a biomarker of aging as it contains isolated cells that are long-lived and not subject to rapid turnover, and as so it fulfils the Baker and Sprott criteria of a biomarker of aging [7] (Chapter 2). Measurement of retinal vascular calibre has already been established as a valid and efficient biomarker of systemic vascular disease [8,9]. The findings from this study suggest that retinal vessel measurement deserves attention as a tool in HIV-related vascular risk estimation (Chapter 7).

13.5 Future work

This study provides a 'snapshot' in time of likely associations in HIV-related accelerated aging. It would be ideal to follow-up this population in a longitudinal study as it is likely that changes in age-related parameters may manifest over a longer time period. The Strategic

Timing of AntiRetroviral Treatment (START) study is an on-going multicentre international trial designed to assess the risks and benefits of initiating ART earlier than is currently practiced [10]; some of the START trial cohorts are based at sites in South Africa. Data from this study will be used to design a prospective study related to clinical, ocular and serum biomarkers of aging, nested within these South African cohorts. It would also be useful to concurrently measure other clinical markers such as neurocognitive function and disability to better understand the health needs of this increasing population.

The study findings also raise further important research questions: prospective assessment of biological age in HIV-infected and HIV-seronegative individuals is needed to ascertain whether the accelerated aging trajectory develops as soon as HIV infection is acquired. Further questions include whether biological age is dependent upon the duration of untreated disease or nadir CD4 count, and if the biological age of the two groups continues to diverge during long-term ART, or rather is modified by ART. In the long-term it will be important to ascertain whether HIV infection accelerates or *accentuates* the aging process. Accelerated aging suggests that aging occurs earlier; however, if aging is not accelerated is it accentuated – i.e. morbidity occurs at the same age, but with higher prevalence? Does HIV both accelerate and accentuate aging? Prospective data with recruitment of both ART-treated and ART-naïve cohorts are required to address these issues.

The need for evaluated biomarkers in determining biological age is gaining increasing importance as data emerge about the excess risk of age-related morbidity in HIV-infected individuals despite suppression of viral load [11]. Several studies have investigated the role of clinical and biochemical biomarkers in determining HIV-related outcomes (Table 1b), however, very few data originate from resource-limited settings or sub-Saharan Africa. Further work is needed to assess the role of these biomarkers in such populations where the aging trajectories in HIV infection may differ compared to well-resourced environments. Stored serum samples are available from this study cohort; further work will involve measurement of e.g. pro-inflammatory cytokines, CRP, D-dimer in this population and relating these to the ocular parameters measured in the current study. Epigenetic markers such as methylation of DNA in peripheral blood leukocytes from this study population will also be evaluated in future work.

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**Appendix 7: Permissions from copyright holders to use manuscripts
within thesis**

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**LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE**

ETHICS COMMITTEE



APPROVAL FORM

Application number: 5889

Name of Principal Investigator Dr. Sophia Pathai

Faculty Infectious and Tropical Diseases

Head of Faculty Professor Simon Croft

Title: Does HIV accelerate the aging process? An assessment of ophthalmic, clinical and serum parameters of aging in a cohort of HIV-infected individuals in South Africa

This application is approved by the Committee.

Chair of the Ethics Committee

Date04 February 2011.....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

UNIVERSITY OF CAPE TOWN



Health Sciences Faculty
Faculty of Health Sciences Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925

Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: sumayah.ariefdien@uct.ac.za

18 February 2011

HREC REF: 011/2011

Dr S Pathai
Desmond Tutu HIV Foundation
IIDMM
FHS

Dear Dr Pathai

PROJECT TITLE: DOES HIV ACCELERATE THE AGING PROCESS? AN ASSESSMENT OF OPHTHALMIC, CLINICAL AND SERUM PARAMETERS OF AGING IN A COHORT OF HIV-INFECTED INDIVIDUALS IN SOUTH AFRICA

Thank you for addressing the concerns raised by the committee.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 28 February 2012.

Please submit a progress form, using the standardised Annual Report Form (FHS016), if the study continues beyond the approval period. Please submit a Standard Closure form (FHS010) if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

A handwritten signature in black ink, appearing to be 'M Blockman', written over a horizontal line.

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

sAriefdien



health

Department:
Health
REPUBLIC OF SOUTH AFRICA

Private Bag X828, PRETORIA, 0001
Civitas Building Corner Andries and Struben Street, PRETORIA, 0001
Tel (012) 395 0922 • Fax (012) _____

Reference : J1/2/4/2 No3/11
Enquiry : Mr JR Mokonoto
Tel : (012) 395-9063
Fax : (086) 632 2606

EXPORT PERMIT

In terms of Section 25 of the Human Tissue Act, 1983 (No 65 of 1983) –

Dr Sophia Pathai
Clinical Research Fellow
Desmond Tutu HIV Centre
Institute for Infectious Diseases & Molecular Medicine
Faculty of Health Sciences
University of Cape Town
Anzio Road, Observatory
CAPE TOWN
7925 South Africa
Tel: 021-650 6970

Fax:-021 650 6963

is hereby authorised to export from the Republic of South Africa –

3 x 740 / 2 ml tubes Blood samples-purified (Integrated) DNA and RNA samples and plasma

from –

Dr Paul Shiels/Prof C Gilbert
Dept of Surgery, University of Glasgow
Level 2, McGregor Building
Western Infirmary, Glasgow
G11 6NT
UNITED KINGDOM
Tel: + 44 0 141 211 2138

Fax: +44 0 141 211 2138

for – Research-To analyse cellular markers of aging (from blood) collaborative project between UCT.

This export permit is subject to the following conditions:

1. The substance shall be imported into the country specified above, within the legal requirements of that country.
2. The substance shall be exported from South Africa and handled in accordance with the provisions of the Human Tissue Act, 1983 (No 65 of 1983), and the regulations made in terms of the Act.
3. The export permit shall not be used for any trade or advertising purposes.
4. **This export permit shall expire on 31 December 2011.**

DIRECTOR-GENERAL: HEALTH
Date: 04.07.2011
Ms P Netshidzivhani

PARTICIPANT INFORMATION SHEET

Study Title:

Does HIV accelerate the aging process? An assessment of ophthalmic, clinical and serum parameters of aging in a cohort of HIV-infected individuals in South Africa.

Who is conducting this study?

Chief investigator: **Dr. Sophia Pathai**

Other Study Staff: Sr. Eudoxia Raditlhalo (079 337 6369)

Please read this form carefully. Take time to ask the study doctor or study staff as many questions about the study as you would like. The study doctor or study staff can explain words or information that you do not understand. Reading this form and talking to the study doctor or study staff may help you decide whether to take part or not. If you decide to take part in this study, you will need to sign your name at the end of this form.

Why is this study being done?

Aging leads to changes in your physical appearance and overall health. Several conditions are linked to age like heart disease, brittle bone disease, kidney disease. It thought that HIV infected persons might be at risk from such conditions at an earlier age, compared to uninfected people of the same age. Similarly, many eye conditions are related to age and this can lead to problems with seeing clearly. This study will assess what kind of age-related eye conditions might be seen in HIV-infected people and if they are different or occur more commonly than in uninfected people. It will also assess other measures relating to age such as physical strength and markers of aging in cells of the body. This study will help us to find out if early aging in HIV infected people does occur. This knowledge can help us to make provision for future healthcare.

Why are you being asked to take part?

You are being asked to take part in this study, so that we can measure the 'amount of aging' in your body in different ways. Your information (data) will be compared with non-infected people of similar age and gender. This will help us find out whether HIV may be related to early aging. Results from this study will help us to understand more about this process.

How many people will take part in the study?

A total of **740 patients** will be recruited for this study. Of these 370 will be HIV patients and 370 will be non-HIV individuals of similar age and sex.

How long will the study last?

This study will last for a period of **1 year**. However, you will not be required to visit the hospital more than once.

What do we do to decide if you are eligible to take part?

We will review clinic records based on a list of factors which suggest you might be suitable for the study (e.g. your age). If you are within this group you will be asked if you would like to participate. If you are interested, the study will be explained to you and you will be allowed to make a decision.

What will happen if you decide to take part in the study?

If you choose to take part,

- You will be asked to **first sign a consent form or give a thumb impression**

Appendix 3a – Version I English

- You will be asked to attend the Department of Ophthalmology at Groote Schuur Hospital for the examination (your travel and time will be reimbursed, see below)
- Your **personal information** (like age, sex, occupation) and your **general medical history** (like heart problems, blood pressure) will be noted.
- We will check your **physical strength and balance** with some simple tests such as rising from a chair and walking along a path.
- Following this, **your eyes will be examined** by an experienced eye doctor
- Your eye doctor will first **check your eye sight**:
 - The vision in each eye will be measured using vision-testing charts.
 - If we find that you need glasses for close vision, these will be given for free
- The **front of your eye** will be examined using a microscope called a “slit-lamp”
- The **back of your eye (retina)** will be examined:
 - Eye drops will be put into each eye, to enlarge the pupil (black circle in middle) of the eye. The eye drops may sting for a few seconds. It can take 20-30 minutes for the eye drops to take effect. If your pupils do not dilate widely enough a second drop may be needed.
 - The drops will enable us to examine the back of the eyes (retina). The examination will involve using a bright light to illuminate the back of your eye. The examination may last 10-15 minutes.
- Some photos will be taken of the back of your eyes using a special camera (fundus camera); *it will not be possible to identify you directly from the photos.* We will also take some measures of the back of the eyes.
- **None of these tests hurt.** Your HIV status will not be disclosed to anyone.
- After the examination you may experience blurring of vision, lights may seem extra bright and you may have difficulty reading or focusing close up. This lasts 2-3 hours and then your eyes and vision will return to normal.
 - In the unlikely event you develop eye pain or more severe blurring of vision you should return to the hospital.
- We will take a **blood sample**.
 - This sample will be taken to measure the degree of aging in the blood cells. To do this we need to extract DNA from cells (DNA is present inside all the cells of your body. It is a code that carries instructions for the form of your body. It is responsible for e.g. the colour of your eyes and hair). This sample will **only** be used to measure aging of cells, and **cannot** be used to determine your complete genetic identity.
 - At the end of the study your sample will be transported to UK for analysis. Only we will use this blood sample, and no one else will have access to it.

What are the risks and discomforts of this study?

The eye drops may cause temporary stinging of the eyes. Very rarely these eye drops will increase your the pressure in your eye, which may cause pain. If this occurs, it is treatable by medication. If you have eye pain after the eye drops are put in your eyes, you should inform us as soon as possible.

Are there any benefits to you for being in the study?

The examination will detect any eye condition, including those related to HIV. The study doctor is an eye doctor. If she detects an eye problem (whether or not related to HIV) she will explain this to you. She will explain the options available; this will probably include arranging an appointment at the hospital eye clinic to see the clinic eye doctors for some further tests and/or treatment. The study doctor can directly refer any eye problem to the eye clinic. You will not need to go back to your community doctor for a referral. The appointment may be on a separate occasion(s) and treatment, if needed, will be provided free. If the hospital eye clinic contacts you to arrange an appointment it will only be via the phone number you have given us. The study doctor will also keep a record of your eye condition, and with your

Appendix 3a – Version I English

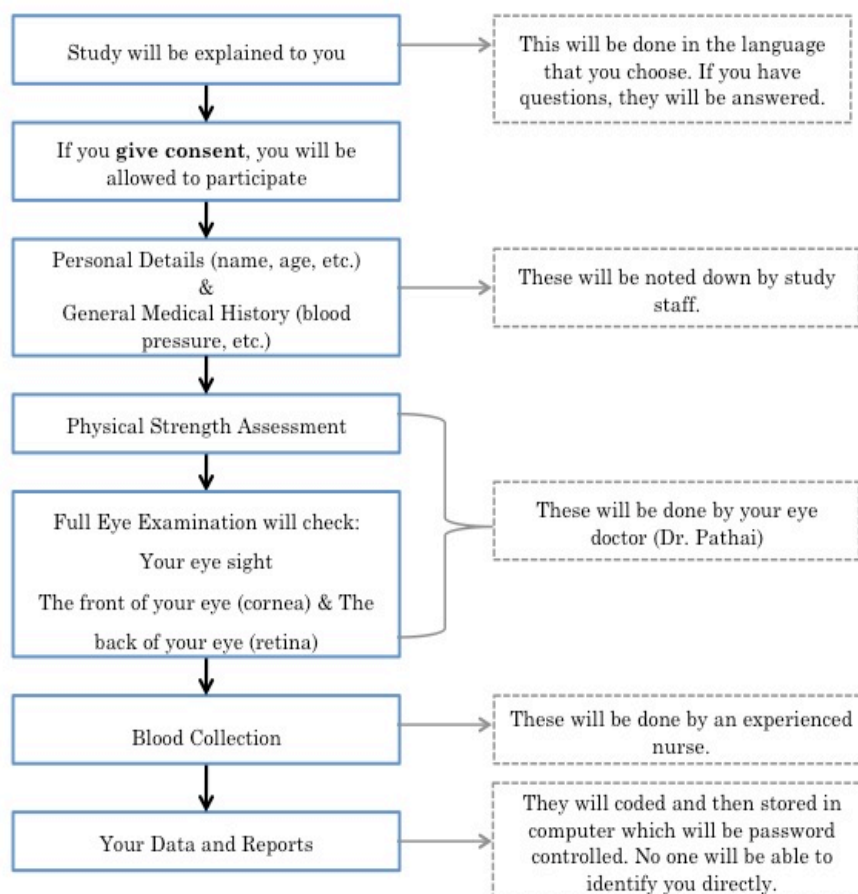
permission will provide this information to the doctors at Hannan Crusaid Clinic. If you need glasses for reading, cooking etc these will be given for free.

What other choices do you have?

You have the following choices:

- You can choose not to take part in the study.
- If you choose to take part, and then wish to dropout (at any point during the study), you may do so. This will not affect your treatment at the clinic.
- If you wish to withdraw your permission for use of your stored blood sample, you may request this. We will make sure that it is destroyed and never used.
- If you do not wish for your identity to be kept with your stored blood samples, you may inform us. We will make sure your identity is kept secret.
- If you do not wish to be contacted after the study is over, for disclosure of any study results, you may request this.

You may refer to the diagrams below to understand the study process better.



What will happen when the study is over?

The clinic staff will receive training on how to detect basic eye problems and will be able arrange referral to the eye clinic as needed. They will also learn how to detect the need for glasses so should you need them in future this will be picked up and you will either be given glasses or an appointment to see an optometrist (optician).

Will your test results be shared with you?

If you would like access to your test results these will be shared with you.

Appendix 3a – Version I English

Will the results of the research be shared with you?

If you would like to have access to the results/ findings of this study after it is completed, they will be shared with you.

Will any of your blood, tissue or other samples be stored and used for research in the future?

You will be asked to give an additional blood sample at the time of your routine testing. The sample will be stored **in a deep freeze**. At the end of the study, samples will be transported to UK for analysis. These analyses will be conducted by the study team laboratory only. No one else will have access to your blood sample, except for study staff. This sample will help us understand aging in HIV patients better. If you do not wish for your identity to be kept with your stored blood sample, it will be kept secret. Only your eye doctor will have access to your full information. If there is any sample remaining this may be stored for research in the future with your permission. Future work will be related to the current project. We will keep your identity anonymous.

Will you receive any reward (money or food vouchers) for taking part in this study?

You will be given a total of ZAR 200, which will cover your travel expenses and time spent participating in the study.

Who will see the information which is collected about you during the study?

- The examination information will be kept confidential and will not be given to anyone outside the study. Your name will only be written on the special enrolment form that has your personal details like name, address, etc. These will be kept in a secure area.
- Your name will never be used in any reports or your HIV status. No quotes or other results arising from your participation in this study will be included in any reports, even anonymously, without your agreement.
- Once your blood sample is transported to UK, no one except for the study doctor will have access to your full information. If you do not want your identity to be kept with your blood sample, it will be removed.
- All study reports/ results will be stored in a computer system. To avoid anyone from identifying you directly all your data will be coded. To ensure that no one outside the study has access to your data, the computer will be password protected. Only the study doctor will have full access.
- If we contact you to provide information or results this will involve only one phone call to the number provided by you. We will not inform anyone else about your HIV status or any other findings.

Who do I speak to (or contact) if I have any questions about the study?

If you have any questions about the study, you can contact the study doctor, Dr. Sophia Pathai or the Research Assistant/Nurse (079 337 6369). In case you require a translator, one will be provided for you.

Before you make a decision, we welcome any questions that you may have.

IPHEPHA LOLWAZI LOMTHATHI-NXAXHEBA

ISihloko soPhononongo: Ingaba i-HIV iyiqhubela phambili imo yokwaluphala? Ukuhlolwa kobunjani obunokubalwa bokwaluphala kwamehlo (ophthalmic), kokugula (clinical) nokwencindi yegazi (serum) kwiqela labantu abosuleleke yi-HIV eMzantsi Afrika.

Ngubani oqhuba olu phononongo?

Umphandi oyintloko: **UGqirha Sophia Pathai**

Esinye iSitafu soPhononongo: Eudoxia Raditlhalo (079 337 6369)

Nceda ufunde le fom ngononophelo. Thatha ixesha ubuze ugqirha wophononongo okanye isitafu sophononongo imibuzo emininzi kangangoko malunga nophononongo kangangoko ufuna. Ugqirha wophononongo okanye isitafu sophononongo bangakuchazela amagama okanye ulwazi ongaluqondiyo. Ukufunda le fom nokuthetha nogqirha wophononongo okanye isitafu sophononongo kungakunceda ukuba wenze isigqibo sokuthatha inxaxheba okanye ungasithathi. Ukuba ugqirha ekubeni uthathe inxaxheba kolu phononongo, kuza kufuneka usayine igama lakho ekupheleni kwale fom.

Kutheni kusenziwa olu phononongo?

Ukwaluphala kungakhokelela kwiinguqulelo kwimbonakalo yomzimba wakho nempilo yakho jikelele. Izigulo ezininzi zinxulunyaniswa nokwaluphala njengesifo sentliziyo, isifo sokubaqwathaqwatha kwamathambo, isifo sezintso. Kucingwa ukuba abantu abosuleleke yi-HIV bangabasemngciphekweni wezigulo ezinjalo besebancinci, xa bethelekiswa nabantu ababudala bufanayo. Ngokufanayo, izigulo zamehlo ezininzi zinxulunyaniswa nokwaluphala yaye oku kungakhokelela kwiingxaki zokungaboni ngokucacileyo. Olu phononongo luza kuhlola ukuba zeziphi izigulo zamehlo ezinxulumene nokwaluphala ezinokubonwa kubantu abosuleleke yi-HIV yaye nokuba ngaba zahlukile na okanye zenzeka ngokuxhaphakileyo na kubantu abangosulelekanga. Luza kuhlola kwakhona amanye amanyathelo anxulumene nokwaluphala anje ngamandla omzimba neziphawuli zokwaluphala kwiiseli zomzimba. Olu phononongo luza kusinceda ukuba sifumanise ukuba ngaba ukwaluphala msinya kubantu abosuleleke yi-HIV kuyenzeka na. Olu lwazi lungasinceda ukuba silungiselele unakekelo lwempilo lwexesha elizayo.

Kutheni ucelwa ukuba uthathe inxaxheba?

Uyacelwa ukuba uthathe inxaxheba kolu phononongo, ukuze sikwazi ukulinganisela 'ubungakanani bokwaluphala' emzimbeni wakho ngendlela ezahlukeneyo. Ulwazi lwakho (idatha) luza kuthelekiswa nolwabantu abangosulelekanga ababudala bulinganayo kwanobuni. Oku kuza kusinceda ukuba sifumanise ukuba ingaba i-HIV inxulumene na nokwaluphala kwamsinya. Iziphumo zolu phononongo ziza kusinceda ukuba siqonde ngokungaphaya malunga nale nkqubo.

Bangaphi abantu abaza kuthatha inxaxheba kuphononongo?

Zizonke **zizigulane ezingama-740** eziza kufunelwa olu phononongo. Kwezi ama-370 aza kuba zizigulane ze-HIV yaye ama-370 aza kuba ngabantu abangekho HIV bobudala obufanayo kwanesini.

Uphononongo luza kuthatha ixesha elingakanani?

Olu phononongo luza kuthatha isithuba **sonyaka om-1**. Nangona kunjalo, awuzukulindeleka ukuba utyelele isibhedlele ngaphezulu kunakanye.

Appendix 3b – Version I Xhosa

Senza ntoni ukwenza isigqibo sokuba ufanelekile na ukuthatha inxaxheba?

Siza kujonga kwakhona iirekhodi zaseklinikhi ngokusekelwe kuluhlu lweemeko ezicebisa ukuba unokululungela uphononongo (umz. Ubudala bakho). Ukuba uphakathi kweli qela uza kubuzwa ukuba ungayithatha na inxaxheba. Ukuba unomdla, uphononongo uza kuluchazelwa yaye uza kuvunyelwa ukuba wenze isigqibo.

Kuza kwenzeka ntoni ukuba ugqiba ekubeni uthathe inxaxheba kuphononongo?

Ukuba ukhetha ukuthatha inxaxheba,

- Uza kucelwa ukuba **uqale usayine ifom yemvume okanye unike uphawu lukabhontsi**
- Uza kucelwa ukuba uye kwiSebe lezaMehlo (Department of Ophthalmology) esiBhedlele iGroote Schuur ukuvavanywa (uhambo lwakho nexesha ziza kuhlalulwa kwakhona, jonga apha ngezantsi)
- **Ulwazi lobuqu** lwakho (njengobudala, isini, isikhundla) kunye **nembali yokugula kwakho jikelele** (njengeengxaki zentliziyo, uxinzelelo lwegazi) ziza kuqwalaselwa.
- Siza kukhangela **amandla omzimba wakho nokuzinza** ngeemvavanyo ezithile ezilula ezinje ngokuphakama esitulweni nokuhamba kwindledlana.
- Ukulandela oku, **amehlo akho aza kuvavanywa** ngugqirha wamehlo onamava
- Ugqirha wakho wamehlo uza kuqala **akhangele ukubona kwakho:**
- Ukubona kwimehlo ngalinye kuza kulinganiswa kusetyenziswa iitshati zokuvavanywa kokubona.
- Ukuba sifumanisa ukuba ufuna iiglaso zokubona kufutshane, ezi ziza kunikwa mahala
- **Umphambili wamehlo lakho** uza kuvavanywa kusetyenziswa ngesibonakhulu (microscope) esibizwa ngokuba sisi-“slit-lamp”
- **Umva wamehlo lakho (iretina)** uza kuvavanywa:
 - Amaqabaza amehlo aza kufakwa kwimehlo ngalinye, ukwandisa ukhozo lwamehlo (isangqa esimnyama esizikithini) semehlo. Amaqabaza amehlo angatsweba imizuzwana embalwa. Kungathatha 20-30 imizuzu ukuba amaqabaza amehlo asebenze. Ukuba iinkozo zamehlo akho azandi ngokubanzi ngokwaneleyo iqabaza lesibini linokufuneka.
 - Amaqabaza aza kusinceda ukuba sivavanye umva wamehlo (iretina). Uvavanyo luza kubandakanya ukusetyenziswa kokukhanya okuqaqambileyo ukukhanyisela umva wamehlo lakho. Uvavanyo lungathatha 10-15 imizuzu.
- Iifoto ezithile zomva wamehlo akho ziza kuthathwa kusetyenziswa ikhamera ekhethekileyo (ikhamera i-fundus); *akuzi kukwazeka ukukubona ngokuthe ngqo kwiifoto*. Siza kuthatha nemilinganiselo ethile kumva wamehlo akho.
- **Akukho nanye kwezi mvavanyo ebuhlungu.** Imeko yakho ye-HIV ayizukudandalaziswa nakubani na.
- Emva kovavanyo ungafumanisa ukubona ngokumfiliba, izibane zingabonakala ziqaqambile yaye kunganzima ukufunda okanye ukujonga kufutshane. Oku kuthatha 2-3 iiyure kuze ke amehlo akho nokubona kubuyele kwisiqhelo.
 - Kwimeko engaqhelekanga uqaqanjelwa limehlo okanye ubona ngokumfiliba okugqithisileyo kufuneka ubuyele esibhedlele.
- Siza kuthatha **isampuli yegazi.**
 - Le sampuli iza kuthathelwa ukulinganisa ubungakanani bokwaluphala kwiiseli zegazi. Ukwenza oku kufuneka sitsale i-DNA kwiiseli (i-DNA ikhona phakathi kweeseli zonke zomzimba wakho. Yikhowudi equlatha imiyalelo yemo yomzimba wakho. Inoxanduva umz. webala lamehlo akho neenwele).

Appendix 3b – Version I Xhosa

Le sampuli iza kusetyenziswa **kuphela** ukulinganisa ukwaluphala kweeseli, yaye **ayinakusetyenziselwa** ukufumanisa ukwaziwa kwakho kofuzo okugqibeleleyo (**complete genetic identity**).

– Ekupheleni kophononongo isampuli yakho iza kuthunyelwa e-UK ukuba ihlalutywe. Sithi kuphela abaza kusebenzisa le sampuli yegazi, yaye akukho mntu ungomnye uza kuba nokufikelela kuyo.

Zeziphi iingozi nokungonwabi kolu phononongo?

Amaqabaza amehlo angabangela ukutsweba kwexeshana emehlweni. Ngokunqabe kakhulu la maqabaza amehlo aza kunyusa uxinzelelo emehlweni lakho, okunokubangela ubuhlungu. Ukuba oku kuyenzeka, kuyanyangeka ngamayeza. Ukuba uyaqaqanjelwa emehlweni emva kokuba amaqabaza amehlo efakwe emehlweni akho, kufuneka usazise ngokukhawuleza kangangoko.

Ingaba kukho naziphi na iinzuzo kuwe ngokuba kuphononongo?

Uvavanyo luza kufumanisa nasiphi na isigulo samehlo, ukuquka nezo zinxulumene ne-HIV. Ugrirha wophononongo ngugqirha wamehlo. Ukuba ufumanisa ingxaki yamehlo (nokuba ngaba inxulumene ne-HIV okanye hayi) uza kukuchazela oku. Uza kukuchazela okukhethwayo okukhoyo; oku mhlawumbi kungaquka ukulucwangcisa idinga kwiklinikhi yamehlo esibhedlele ukubona oogqirha bamehlo baseklinikhi ngeemvavanyo ezingaphaya kunye/okanye unyango. Ugqirha wophononongo angadlulisela ngokuthe ngqo nayiphi na ingxaki yamehlo kwiklinikhi yamehlo. Akuzi kufuneka ukuba ubuyeke kugqirha wakho wengingqi ngodluliselo. Idinga linokuba nge(ngama)xesha elo(awo)hlukeneyo yaye unyango, ukuba luyafuneka, luza kunikwa mahala. Ukuba iklinikhi yamehlo yesibhedlele iqhagamshelana nawe ukucwangcisa idinga kuya kuba kuphela ngenombolo yomnxeba osinike yona. Ugqirha wophononongo uza kugcina kwakhona irekhodi yesigulo sakho samehlo, yaye ngemvume yakho uza kunikeza olu lwazi koogqirha kwi-Hannan Crusaid Clinic. Ukuba ufuna iiglaszi zokufunda, ukupheka njl.njl ziya kunikwa mahala.

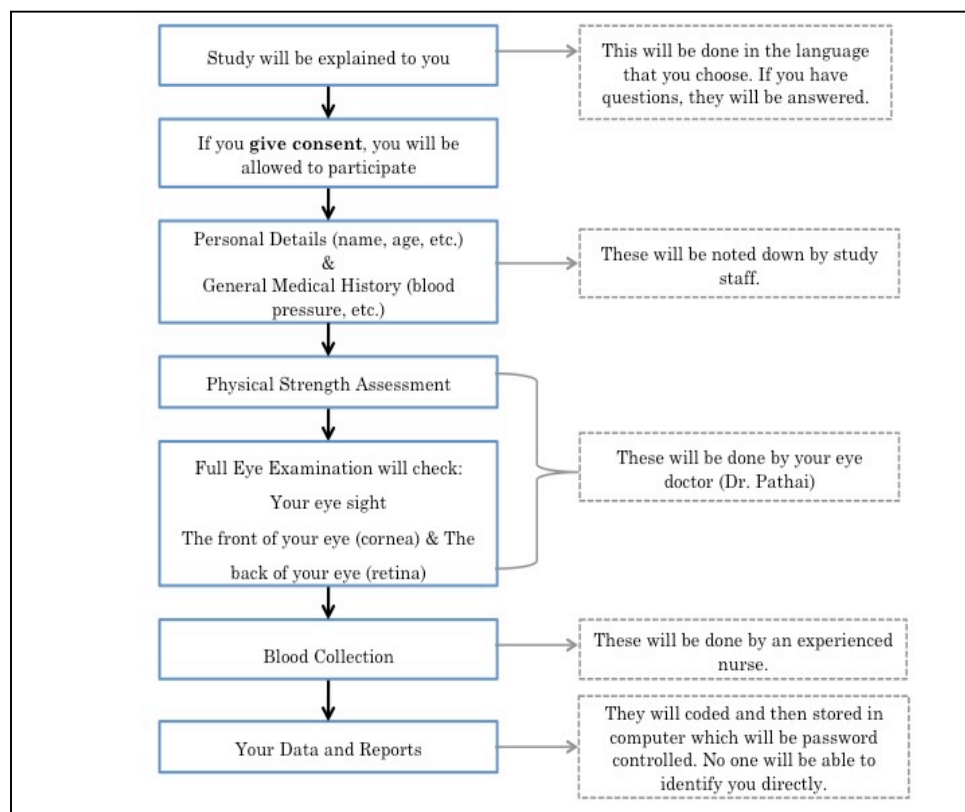
Kokuphi ukukhetha okukokunye onako?

Unoku kukhetha kulandelayo:

- Ungakhetha ukungathathi nxaxheba kuphononongo.
- Ukuba ukhetha ukuthatha inxaxheba, uze ke unqwenele ukurhoxa (nanini na ngexesha lophando), ungenza njalo. Oku akuzi kuchaphazela unyango lwakho eklinikhi.
- Ukuba unqwanela ukurhoxisa imvume yakho yokusetyenziswa kwesampuli yegazi yakho egciniweyo, ungakucela oku. Siza kuqinisekisa ukuba iyatshatyalaliswa yaye ayisoze isetyenziswe.
- Ukuba awunqwenele ukuba ukwaziwa kwakho kugcinwe neesampuli zegazi lakho, ungasazisa. Siza kuqinisekisa ukuba ukwaziwa kwakho kugcinwa kuyimfihlo.
- Ukuba awunqwenele ukuba kuqhagamshelwane nawe emva kokuba uphononongo luphelile, ngokudandalaziswa kwazo naziphi na iziphumo zophononongo, ungakucela oku.

Ungajonga kwiidayagram apha ngezantsi ukuqonda inkqubo yophononongo ngokungcono.

Appendix 3b – Version I Xhosa



Kuza kwenzeka ntoni lusakube uphononongo luphelile?

Isitafu saseklinikhi siza kufumana uqeqesho ngendlela yokufumanisa iingxaki zamehlo ezingundoqo yaye siza kubanako ukucwangcisa udluliselo kwiklinikhi yamehlo njengoko kufunwa. Baza kufunda nendlela yokufumanisa imfuneko yeeglasi ngako oko xa unokuthi uzifune kwixesha elizayo oku kuza kufunyaniswa yaye uza kuthi mhlawumbi ufumane iiglasi okanye idinga lokubona ugqirha wamehlo (ingcali yamehlo).

Ingaba iziphumo zovavanyo zakho kuza kwabelwana ngazo nawe?

Ukuba uyakufuna ukufikelela kwiziphumo zakho zovavanyo kuza kwabelwana ngazo nawe.

Ingaba iziphumo zophando kuza kwabelwana ngazo nawe?

Ukuba uyakufuna ukufikelela kwiziphumo/ izinto ezifunyanisiweyo zolu phononongo emva kokuba lugqityiwe, kuza kwabelwana ngazo nawe.

Ingaba naziphi na iisampuli zakho zegazi, isihlunu okanye naziphi na iisampuli ezizezinye ziza kugcinwa zize zisetyenziselwe uphando kwixesha elizayo?

Uza kucelwa ukuba unike isampuli yegazi eyongezelelweyo ngexesha lovavanyo lwakho lwesiqhelo. Isampuli iza kugcinwa **kwisikhenkcisi esibandisa kakhulu (deep freeze)**. Ekupheleni kophononongo, iisampuli ziza kuthunyelwa e-UK ukuba zihlalutywe. Olu hlalutyo luza kuqhutywa yilebhu yeqela lophando kuphela. Akukho mntu wumbi uza kufikelela kwisampuli yakho yegazi, ngaphandle kwesitafu sophononongo. Le sampuli iza kusinceda ukuqonda ukwaluphala kwizigulane ze-HIV ngokungcono. Ukuba awunqweneli ukuba ukwaziwa kwakho kugcinwe nesampuli yegazi yakho, oku kuza kugcinwa ngokuyimfihlo. Ngugqirha wakho wamehlo kuphela oza kufikelela kulwazi lwakho olupheleleyo. Ukuba kukho nayiphi na isampuli eseleyo inokuthi igcinelwe uphando lwexesha elizayo ngemvume yakho. Umsebenzi wexesha elizayo uza kunxulumana neprojekthi yangoku. Siza kugcina ukwaziwa kwakho kungaziwa.

Appendix 3b – Version I Xhosa

Uza kufumana nawuphi na umvuzo (imali okanye iivawutsha zokutya) ngokuthatha inxaxheba kolu phononongo?

Uza kunikwa iyonke i-ZAR 200, eza kuhlawula iindleko zohambo zakho nexesha olichithileyo ngokuthatha inxaxheba kuphononongo.

Ngubani oza kubona ulwazi oluqokelelweyo ngawe ngexesha lophononongo?

- Ulwazi lovavanyo luza kugcinwa luyimfihlo yaye aluzi kunikwa nabani na ongaphandle kophononongo. Igama lakho liza kubhalwa kuphela kwifom yobhaliso olukhethekileyo eneenkcukacha zakho zobuqu ezinje ngegama, idilesi, njl.njl. Ezi ziza kugcinwa kwindawo ekhuselekileyo.
- Igama lakho alisoze lisetyenziswe nakwiziphina iingxelo okanye isimo sakho se-HIV. Akukho manqaku okanye ezinye iziphumo ezivela ekuthabatheni kwakho inxaxheba kolu phononongo ziya kuthi ziqukwe kuzo naziphi na iingxelo, nokuba ngaba kungokungaziwayo, ngaphandle kwemvume yakho.
- Ukuba nje isampuli yegazi yakho ithunyelwe e-UK, akukho nabani na ngaphandle koggirha wophononongo oya kuthi afikelele kulwazi lwakho olupheleleyo. Ukuba awufuni ukuba ukwaziwa kwakho kugcinwe nesampuli yegazi yakho, kuza kususwa.
- Zonke iingxelo/iziphumo zophando ziza kugcinwa kwinkqubo yekhompyutha. Ukunqanda nabani na ukuba akwazi ngokungqalileyo yonke idatha yakho iza kukhowudwa. Ukuqinisekisa ukuba akukho nabani na ongaphandle oya uthi afikelele kwidatha yakho, ikhompyutha iza kukhuselwa nge-password. Ngugqirha wophando kuphela oza kubanokufikelela okupheleleyo.
- Ukuba siqhagamshelana nawe ukukunika ulwazi okanye iziphumo oku kuya kubandakanya utsalo lomnxeba olunye kuphela kwinombolo enikwe nguwe. Asizukwazisa nabani na ongomnye malunga nesimo sakho se-HIV okanye naziphi na izinto ezifunyanisiweyo.

Ndithetha nabani (okanye uqhagamshelwano) ukuba ndinayo nayiphi na imibuzo malunga nophononongo?

Ukuba unayo nayiphi na imibuzo malunga nophononongo, ungaqhagamshelana nogqirha wophononongo, uGqirha Sophia Pathai okanye iSekela loPhando/uNesi (079 337 6369). Xa unokuthi ufune umguquli, uza kumnikwa.

Phambi kokuba wenze isigqibo, samkela nayiphi na imibuzo onokuthi ube nayo.

PARTICIPANT INFORMATION SHEET

Study Title:

Does HIV accelerate the aging process? An assessment of ophthalmic, clinical and serum parameters of aging in a cohort of HIV-infected individuals in South Africa.

Who is conducting this study?

Chief investigator: **Dr. Sophia Pathai**

Other Study Staff: Sr Eudoxia Radithalo (079 337 6369)

Please read this form carefully. Take time to ask the study doctor or study staff as many questions about the study as you would like. The study doctor or study staff can explain words or information that you do not understand. Reading this form and talking to the study doctor or study staff may help you decide whether to take part or not. If you decide to take part in this study, you will need to sign your name at the end of this form.

Why is this study being done?

Aging leads to changes in your physical appearance and overall health. Several conditions are linked to age like heart disease, brittle bone disease, kidney disease. It thought that HIV infected persons might be at risk from such conditions at an earlier age, compared to uninfected people of the same age. Similarly, many eye conditions are related to age and this can lead to problems with seeing clearly. This study will assess what kind of age-related eye conditions might be seen in HIV-infected people and if they are different or occur more commonly than in uninfected people. It will also assess other measures relating to age such as physical strength and markers of aging in cells of the body. This study will help us to find out if early aging in HIV infected people does occur. This knowledge can help us to make provision for future healthcare.

Why are you being asked to take part?

You are being asked to take part in this study, so that we can measure the 'amount of aging' in your body in different ways. Your information (data) will be compared with HIV-infected people of similar age and gender – therefore you will be used as a 'comparison'. This will help us find out whether HIV may be related to early aging. Results from this study will help us to understand more about this process. Your participation is greatly valued as we need to examine a comparison group who are not infected with HIV. We will use pre-existing information from the Emavundleni Clinic and you will not be required to undergo any additional HIV tests.

How many people will take part in the study?

A total of **740 patients** will be recruited for this study. Of these 370 will be HIV patients and 370 will be non-HIV individuals of similar age and sex.

How long will the study last?

This study will last for a period of **1 year**. However, you will not be required to visit the hospital more than once.

What do we do to decide if you are eligible to be take part?

We will review clinic records based on a list of factors which suggest you might be suitable for the study (e.g. your age). If you are within this group you will be asked if you would like to participate. If you are interested, the study will be explained to you and you will be allowed to make a decision.

What will happen if you decide to take part in the study?

Appendix 3c – Version 2 (HIV-uninfected) English

If you choose to take part,

- You will be asked to **first sign a consent form or give a thumb impression**
- You will be asked to attend the Department of Ophthalmology at Groote Schuur Hospital for the examination (your travel and time will be reimbursed, see below)
- Your **personal information** (like age, sex, occupation) and your **general medical history** (like heart problems, blood pressure) will be noted.
- We will check your **physical strength and balance** with some simple tests such as rising from a chair and walking along a path.
- Following this, **your eyes will be examined** by an experienced eye doctor
- Your eye doctor will first **check your eye sight**, which will be done as follows:
 - The vision in each eye will be measured using vision-testing charts.
 - If we find that you need glasses for close vision, these will be given for free.
- The **front of your eye** will be examined using a special microscope called a “slit-lamp”
- The **back of your eye (retina)** will be examined as follows:
 - Eye drops will be put into each eye, to enlarge the pupil (black circle in middle) of the eye. The eye drops may sting for a few seconds. It can take 20-30 minutes for the eye drops to take effect. If your pupils do not dilate widely enough a second drop may be needed.
 - The drops will enable us to examine the back of the eyes (retina). The examination will involve using a bright light to illuminate the back of your eye. The examination may last 10-15 minutes.
- Some photos will be taken of the back of your eyes using a special camera (fundus camera); *it will not be possible to identify you directly from the photos.* We will also take some measures of the back of the eyes.
- **None of these tests hurt.** We will make sure that the eye examination is as quick and pain free as possible.
- After the examination you may experience blurring of vision, lights may seem extra bright and you may have difficulty reading or focusing up close. This lasts 2-3 hours and then your eyes and vision will return to normal.
 - In the unlikely event that you develop eye pain or more severe blurring of vision you should return to the hospital.
- We will take a **blood sample**.
 - This sample will be taken to measure the degree of aging in the blood cells. To do this we need to extract DNA from cells (DNA is present inside all the cells of your body. It is a code that carries instructions for the form of your body. It is responsible for e.g. the colour of your eyes and hair). This sample will **only** be used to measure aging of cells, and **cannot** be used to determine your complete genetic identity.
 - At the end of the study your sample will be transported to UK for analysis. Only we will use this blood sample, and no one else will have access to it.

What are the risks and discomforts of this study?

The eye drops may cause temporary stinging of the eyes. Very rarely these eye drops will increase your the pressure in your eye, which may cause pain. If this occurs, it is treatable by medication. If you have eye pain after the eye drops are put in your eyes, you should inform us as soon as possible.

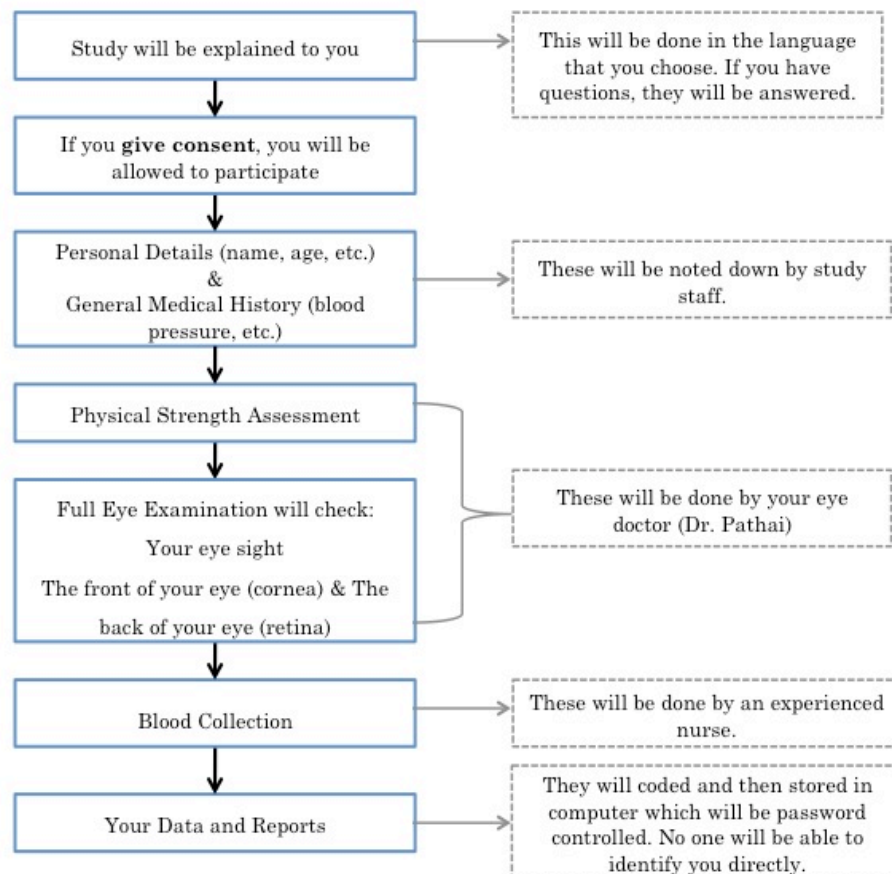
Are there any benefits to you for being in the study?

The examination will detect any eye condition you may have. The study doctor is an eye doctor. If she detects an eye problem she will explain this to you. She will explain the options available; this will probably include arranging an appointment at the hospital eye clinic to see the clinic eye doctors for some further tests and/or treatment. The study doctor can directly refer any eye problem to the eye clinic. You

Appendix 3c – Version 2 (HIV-uninfected) English

will not need to go back to your community doctor for a referral. The appointment may be on a separate occasion(s) and treatment, if needed, will be provided free. If the hospital eye clinic contacts you to arrange an appointment it will only be via the phone number you have given us. The study doctor will also keep a record of your eye condition, and with your permission will provide this information to your community doctor. If you need glasses for reading, cooking etc these will be given for free.

You may refer to the diagrams below to understand the study better



What other choices do you have?

You have the following choices:

- You can choose not to take part in the study.
- If you choose to take part, and then wish to dropout (at any point during the study), you may do so. This will not affect your current or future treatment or management at the clinic.
- If you wish to withdraw your permission for use of your stored blood sample, you may request this. We will make sure that it is destroyed and never used.
- If you do not wish for your identity to be kept with your stored blood samples, you may inform us. We will make sure your identity is kept secret.
- If you do not wish to be contacted after the study is over, for disclosure of any study results, you may request this.

What will happen when the study is over?

Appendix 3c – Version 2 (HIV-uninfected) English

Once the study is over you will be able to access eye care services as usual. We will train the clinic staff to detect basic eye conditions and the need for glasses so this can be picked up if you have any eye-related symptoms.

Will your test results be shared with you?

If you would like access to your test results these will be shared with you.

Will the results of the research be shared with you?

If you would like to have access to the results/ findings of this study after it is completed, they will be shared with you.

Will any of your blood, tissue or other samples be stored and used for research in the future?

You will be asked to give an additional blood sample at the time of your routine testing. The sample will be stored **in a deep freeze**. At the end of the study, samples will be transported to UK for analysis. These analyses will be conducted by the study team laboratory only. No one else will have access to your blood sample, except for study staff. This sample will help us understand aging in cells better. At the end of the analysis, if you choose to have access to these results, they will be shared with you. If you do not wish for your identity to be kept with your stored blood sample, it will be kept secret. Only your eye doctor will have access to your full information. If there is any sample remaining this may be stored for research in the future with your permission. Future work will be related to this project. We will keep your identity anonymous.

Will you receive any reward (money or food vouchers) for taking part in this study?

You will be given a total of ZAR 200, which will cover your travel expenses and time spent participating in the study.

Who will see the information which is collected about you during the study?

- The examination information will be kept confidential and will not be given to anyone outside the study. Your name will only be written on the special enrolment form that has your personal details like name, address, etc. These will be kept in a secure area.
- Your name will never be used in any reports. No quotes or other results arising from your participation in this study will be included in any reports, even anonymously, without your agreement.
- Once your blood sample is transported to UK, no one except for the study doctor will have access to your full information. If you do not want your identity to be kept with your blood sample, it will be removed.
- All study reports/ results will be stored in a computer system. To avoid anyone from identifying you directly all your data will be coded. To ensure that no one outside the study has access to your data, the computer will be password protected. Only the study doctor will have full access.
- If we contact you to provide information or results this will involve only one phone call to the number provided by you.

Who do I speak to (or contact) if I have any questions about the study?

If you have any questions about the study, you can contact your the study doctor, Dr. Sophia Pathai or the Research Assistant/Nurse (079 337 6369). In case you require a translator, one will be provided for you.

Before you make a decision, we welcome any questions that you may have.

Appendix 3d – Version 2 (HIV-uninfected) Xhosa

IPHEPHA LOLWAZI LOMTHATHI-NXAXHEBA

ISihloko soPhononongo:

Ingaba i-HIV iyiqhubela phambili imo yokwaluphala? Ukuhlolwa kobunjani obunokubalwa bokwaluphala kwamehlo (ophthalmic), kokugula (clinical) nokwencindi yegazi (serum) kwiqela labantu abosuleleke yi-HIV eMzantsi Afrika.

Ngubani oqhuba olu phononongo?

Umphandi oyintloko: **UGqirha Sophia Pathai**

Esinye iSitafu soPhononongo: (Sr Eudoxia Radithalo)

Nceda ufunde le fom ngononophelo. Thatha ixesha ubuze ugqirha wophononongo okanye isitafu sophononongo imibuzo emininzi kangangoko malunga nophononongo kangangoko ufuna. Ugqirha wophononongo okanye isitafu sophononongo bangakuchazela amagama okanye ulwazi ongaluqondiyo. Ukufunda le fom nokuthetha nogqirha wophononongo okanye isitafu sophononongo kungakunceda ukuba wenze isigqibo sokuthatha inxaxheba okanye ungasithathi. Ukuba ugqirha ekubeni uthathe inxaxheba kolu phononongo, kuza kufuneka usayine igama lakho ekupheleni kwale fom.

Kutheni kusenziwa olu phononongo?

Ukwaluphala kungakhokelela kwiinguqulelo kwimbonakalo yomzimba wakho nempilo yakho jikelele. Izigulo ezininzi zinxulunyaniswa nokwaluphala njengesifo sentliziyo, isifo sokubaqwathaqwatha kwamathambo, isifo sezintso. Kucingwa ukuba abantu abosuleleke yi-HIV bangabasemngciphekweni wezigulo ezinjalo besebancinci, xa bethelekiswa nabantu ababudala bufanayo. Ngokufanayo, izigulo zamehlo ezininzi zinxulunyaniswa nokwaluphala yaye oku kungakhokelela kwiingxaki zokungaboni ngokucacileyo. Olu phononongo luza kuhlola ukuba zeziphi izigulo zamehlo ezinxulumene nokwaluphala ezinokubonwa kubantu abosuleleke yi-HIV yaye nokuba ngaba zahlukile na okanye zenzeka ngokuxhaphakileyo na kubantu abangosulelekanga. Luza kuhlola kwakhona amanye amanyathelo anxulumene nokwaluphala anje ngamandla omzimba neziphawuli zokwaluphala kwiiseli zomzimba. Olu phononongo luza kusinceda ukuba sifumanise ukuba ngaba ukwaluphala msinya kubantu abosuleleke yi-HIV kuyenzeka na. Olu lwazi lungasinceda ukuba silungiselele unakekelo lwempilo lwexesha elizayo.

Kutheni ucelwa ukuba uthathe inxaxheba?

Uyacelwa ukuba uthathe inxaxheba kolu phononongo, ukuze sikwazi ukulinganisela 'ubungakanani bokwaluphala' emzimbeni wakho ngendlela ezahlukeneyo. Ulwazi lwakho (idatha) luza kuthelekiswa nolwabantu abosuleleke yi-HIV ababudala bulinganayo kwanobuni –ngoko ke uza kusetyenziswa 'njengothelekiso'. Oku kuza kusinceda ukuba sifumanise ukuba ingaba i-HIV inxulumene na nokwaluphala kwamsinya. Iziphumo zolu phononongo ziza kusinceda ukuba siqonde ngokungaphaya malunga nale nkqubo. Ukuthatha kwakho inxaxheba kuxabiseke kakhulu nanjengoko kufuneka sihlale iqela lothelekiso elingosulelekanga yi-HIV. Siza kusebenzisa ulwazi obelusele lukhona kwangaphambili kwiKlinikhi yaseMavundleni (Emavundleni Clinic) yaye awuzukulindeleka ukuba wenziwe naziphi na iimvavanyo ze-HIV ezongezelelweyo.

Bangaphi abantu abaza kuthatha inxaxheba kuphononongo?

Zizonke zizigulane ezingama-740 eziza kufunelwa olu phononongo. Kwezi ama-370 aza kuba zizigulane ze-HIV yaye ama-370 aza kuba ngabantu abangekho HIV bobudala obufanayo kwanesini.

Uphononongo luza kuthatha ixesha elingakanani?

Appendix 3d – Version 2 (HIV-uninfected) Xhosa

Olu phononongo luza kuthatha isithuba **sonyaka om-1**. Nangona kunjalo, awuzukulindeleka ukuba utyelele isibhedlele ngaphezulu kunakanye.

Senza ntoni ukwenza isigqibo sokuba ufanelekile na ukuthatha inxaxheba?

Siza kujonga kwakhona iirekhodi zaseklinikhi ngokusekelwe kuluhlu lweemeko ezicebisa ukuba unokulungela uphononongo (umz. Ubudala bakho). Ukuba uphakathi kweli qela uza kubuzwa ukuba ungayithatha na inxaxheba. Ukuba unomdla, uphononongo uza kuluchazelwa yaye uza kuvunyelwa ukuba wenze isigqibo.

Kuza kwenzeka ntoni ukuba ugqiba ekubeni uthathe inxaxheba kuphononongo?

Ukuba ukhetha ukuthatha inxaxheba,

- Uza kucelwa ukuba **uqale usayine ifom yemvume okanye unike uphawu lukabhontsi**
- Uza kucelwa ukuba uye kwiSebe lezaMehlo (Department of Ophthalmology) esiBhedlele iGroote Schuur ukuvavanywa (uhambo lwakho nexesha ziza kuhlawulwa kwakhona, jonga apha ngezantsi)
- **Ulwazi lobuqu** lwakho (njengobudala, isini, isikhundla) kunye **nembali yokugula kwakho jikelele** (njengeengxaki zentliziyo, uxinzelelo lwegazi) ziza kuqwalaselwa.
- Siza kukhangela **amandla omzimba wakho nokuzinza** ngeemvavanyo ezithile ezilula ezinje ngokuphakama esitulweni nokuhamba kwindledlana.
- Ukulandela oku, **amehlo akho aza kuvavanywa** ngugqirha wamehlo
- Ugqirha wakho wamehlo uza kuqala **akhangele ukubona kwakho**, okuza kwenziwa ngale ndlela ilandelayo:
 - Ukubona kwimehlo ngalinye kuza kulinganiswa kusetyenziswa iitshati zokuvavanywa kokubona.
 - Ukuba sifumanisa ukuba ufuna iiglaszi zokubona kufutshane, ezi ziza kunikwa mahala
 - **Umphambili wamehlo lakho** uza kuvavanywa kusetyenziswa ngesibonakhulu esikhethekileyo (microscope) esibizwa ngokuba sisi-“slit-lamp”
 - **Umva wamehlo lakho (iretina)** uza kuvavanywa ngale ndlela ilandelayo:
 - Amaqabaza amehlo aza kufakwa kwimehlo ngalinye, ukwandisa ukhozo lwamehlo (isangqa esimnyama esizikithini) semehlo. Amaqabaza amehlo angatsweba imizuzwana embalwa. Kungathatha 20-30 imizuzu ukuba amaqabaza amehlo asebenze. Ukuba iinkozo zamehlo akho azandi ngokubanzi ngokwaneleyo iqabaza lesibini linokufuneka.
 - Amaqabaza aza kusinceda ukuba sivavanye umva wamehlo (iretina). Uvavanyo luza kubandakanya ukusetyenziswa kokukhanya okuqaqambileyo ukukhanyisela umva wamehlo lakho. Uvavanyo lungathatha 10-15 imizuzu.
 - lifoto ezithile zomva wamehlo akho ziza kuthathwa kusetyenziswa ikhamera ekhethekileyo (ikhamera i-fundus); *akuzi kukwazeka ukukubona ngokuthe ngqo kwiifoto*. Siza kuthatha nemilinganiselo ethile kumva wamehlo akho.
 - **Akukho nanye kwezi mvavanyo ebuhlungu.** Siza kuqinisekisa ukuba ukuxilongwa kwamehlo akho kukhawuleza yaye akukho buhlungu kangangoko.
 - Emva kovavanyo ungafumanisa ukubona ngokumfiliba, izibane zingabonakala ziqaqambile yaye kunganzima ukufunda okanye ukujonga kufutshane. Oku kuthatha 2-3 iiyure kuze ke amehlo akho nokubona kubuyele kwisiqhelo.
 - Kwimeko engaqhelekanga yokuba uqaqanjelwe limehlo okanye ubone ngokumfiliba okugqithisileyo kufuneka ubuyele esibhedlele.
 - Siza kuthatha **isampuli yegazi**.

Appendix 3d – Version 2 (HIV-uninfected) Xhosa

- Le sampuli iza kuthathelwa ukulinganisa ubungakanani bokwaluphala kwiiseli zegazi. Ukwenza oku kufuneka sitsale i-DNA kwiiseli (i-DNA ikhona phakathi kweeseli zonke zomzimba wakho. Yikhowudi equlatha imiyalelo yemo yomzimba wakho. Inoxanduva (umz. webala lamehlo akho neenwele). Le sampuli iza kusetyenziswa **kuphela** ukulinganisa ukwaluphala kweeseli, yaye **ayinakusetyenziselwa** ukufumanisa ukwaziwa kwakho kofuzo okugqibeleleyo (**complete genetic identity**).
- Ekupheleni kophononongo isampuli yakho iza kuthunyelwa e-UK ukuba ihlalutywe. Sithi kuphela abaza kusebenzisa le sampuli yegazi, yaye akukho mntu ungomnye uza kuba nokufikelela kuyo.

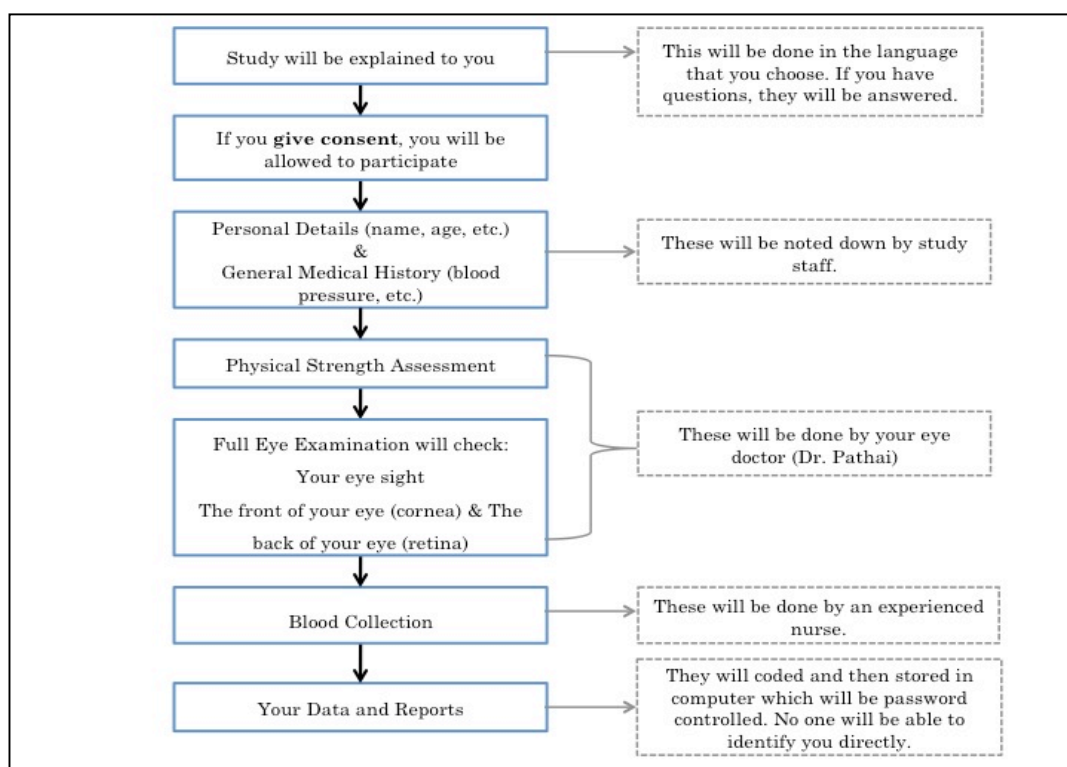
Zeziphi iingozi nokungonwabi kolu phononongo?

Amaqabaza amehlo angabangela ukutsweba kwexeshana emehlweni. Ngokunqabe kakhulu la maqabaza amehlo aza kunyusa uxinzelelo emehlweni lakho, okunokubangela ubuhlungu. Ukuba oku kuyenzeka, kuyanyangeka ngamayeza. Ukuba uyaqaqanjelwa emehlweni emva kokuba amaqabaza amehlo efakwe emehlweni akho, kufuneka usazise ngokukhawuleza kangangoko.

Ingaba kukho naziphi na iinzuzo kuwe ngokuba kuphononongo?

Uvavanyo luza kufumanisa nasiphi na isigulo samehlo onokuba unaso. Ugrirha wophononongo ngugqirha wamehlo. Ukuba ufumanisa ingxaki yamehlo uza kukuchazela oku. Uza kukuchazela okukhethwayo okukhoyo; oku mhlawumbi kungaquka ukulucwangcisa idinga kwiklinikhi yamehlo esibhedlele ukubona oogqirha bamehlo baseklinikhi ngeemvavanyo ezingaphaya kunye/okanye unyango. Ugqirha wophononongo angadlulisela ngokuthe ngqo nayiphi na ingxaki yamehlo kwiklinikhi yamehlo. Akuzi kufuneka ukuba ubuyele kugqirha wakho wengingqi ngodluliselo. Idinga linokuba nge(ngama)xesha elo(awo)hlukeneyo yaye unyango, ukuba luyafuneka, luza kunikwa mahala. Ukuba iiklinikhi yamehlo yesibhedlele iqhagamshelana nawe ukucwangcisa idinga kuya kuba kuphela ngenombolo yomnxeba osinike yona. Ugqirha wophononongo uza kugcina kwakhona irekhodi yesigulo sakho samehlo, yaye ngemvume yakho uza kunikeza olu lwazi kugqirha wakho wengingqi. Ukuba ufuna iiglaszi zokufunda, ukupheka njl.njl ziya kunikwa mahala.

Ungajonga kwiidayagram apha ngezantsi ukuqonda inkqubo yophononongo ngokungcono.



Appendix 3d – Version 2 (HIV-uninfected) Xhosa

Kokuphi ukukhetha okukokunye onako?

Unoku kukhetha kulandelayo:

- Ungakhetha ukungathathi nxaxheba kuphononongo.
- Ukuba ukhetha ukuthatha inxaxheba, uze ke unqwenele ukurhoxa (nanini na ngexesha lophando), ungenza njalo. Oku akuzi kuchaphazela unyango lwakho lwangoku okanye lwexesha elizayo okanye ulawulo lwalo eklinikhi.
- Ukuba unqwenele ukurhoxisa imvume yakho yokusetyenziswa kwesampuli yegazi yakho egciniweyo, ungakucela oku. Siza kuqinisekisa ukuba iyatshatyalaliswa yaye ayisoze isetyenziswe.
- Ukuba awunqwenele ukuba ukwaziwa kwakho kugcinwe neesampuli zegazi lakho, ungasazisa. Siza kuqinisekisa ukuba ukwaziwa kwakho kugcinwa kuyimfihlo.
- Ukuba awunqwenele ukuba kuqhagamshelwane nawe emva kokuba uphononongo luphelile, ngokudandalaziswa kwazo naziphi na iziphumo zophononongo, ungakucela oku.

Kuza kwenzeka ntoni lusakube uphononongo luphelile?

Ukuba nje uphando lugqitywe uza kubanako ukufikelela kwiinkonzo zonakekelo lwamehlo njengesiqhelo. Siza kuqeqesha isitafu saseklinikhi ukufumanisa izigulo zamehlo ezingundoqo kunye nemfuneko yeeglasu ukuze kufunyaniswe ukuba unazo naziphi na iimpawu ezinxulumene namehlo.

Ingaba iziphumo zovavanyo zakho kuza kwabelwana ngazo nawe?

Ukuba uyakufuna ukufikelela kwiziphumo zakho zovavanyo kuza kwabelwana ngazo nawe.

Ingaba iziphumo zophando kuza kwabelwana ngazo nawe?

Ukuba uyakufuna ukufikelela kwiziphumo/ izinto ezifunyanisiweyo zolu phononongo emva kokuba lugqitywe, kuza kwabelwana ngazo nawe.

Ingaba naziphi na iisampuli zakho zegazi, isihlunu okanye naziphi na iisampuli ezizezinye ziza kugcinwa zize zisetyenziselwe uphando kwixesha elizayo?

Uza kucelwa ukuba unike isampuli yegazi eyongezelelweyo ngexesha lovavanyo lwakho lwesiqhelo. Isampuli iza kugcinwa **kwisikhenkcisi esibandisa kakhulu (deep freeze)**. Ekupheleni kophononongo, iisampuli ziza kuthunyelwa e-UK ukuba zihlalutywe. Olu hlalutywe luza kuqhutywa yilebhu yeqela lophando kuphela. Akukho mntu wumbi uza kufikelela kwisampuli yakho yegazi, ngaphandle kwesitafu sophononongo. Le sampuli iza kusinceda ukuqonda ukwaluphala kweeseli ngokungcono. Ekupheleni kohlalutywe, ukuba ukhetha ukufikelela kwezi ziphumo, kuza kwabelwana ngazo nawe. Ukuba awunqwenele ukuba ukwaziwa kwakho kugcinwe nesampuli yegazi yakho, oku kuza kugcinwa ngokuyimfihlo. Ngugqirha wakho wamehlo kuphela oza kufikelela kulwazi lwakho olupheleleyo. Ukuba kukho nayiphi na isampuli eseleyo inokuthi igcinelwe uphando lwexesha elizayo ngemvume yakho. Umsebenzi wexesha elizayo uza kunxulumana nale projekthi. Siza kugcina ukwaziwa kwakho kungaziwa.

Uza kufumana nawuphi na umvuzo (imali okanye iivawutsha zokutya) ngokuthatha inxaxheba kolu phononongo?

Uza kunikwa iyonke i-ZAR 200, eza kuhlawula iindleko zohambo zakho nexesha olichithileyo ngokuthatha inxaxheba kuphononongo.

Ngubani oza kubona ulwazi oluqokelelweyo ngawe ngexesha lophononongo?

- Ulwazi lovavanyo luza kugcinwa luyimfihlo yaye aluzi kunikwa nabani na ongaphandle kophononongo. Igama lakho liza kubhalwa kuphela kwifom

Appendix 3d – Version 2 (HIV-uninfected) Xhosa

yobhaliso olukhethekileyo eneenkcukacha zakho zobuqu ezinje ngegama, idilesi, njl.njl. Ezi ziza kugcinwa kwindawo ekhuselekileyo.

- Igama lakho alisoze lisetyenziswe nakwiziphina iingxelo. Akukho zingxelo okanye ezinye iziphumo ezivela ekuthabatheni kwakho inxaxheba kolu phononongo ziya kuthi ziqukwe kuzo naziphi na iingxelo, nokuba ngaba kungokungaziwayo, ngaphandle kwemvume yakho.
- Ukuba nje isampuli yegazi yakho ithunyelwe e-UK, akukho nabani na ngaphandle kogqirha wophononongo oya kuthi afikelele kulwazi lwakho olupheleleyo. Ukuba awufuni ukuba ukwaziwa kwakho kugcinwe nesampuli yegazi yakho, kuza kususwa.
- Zonke iingxelo/iziphumo zophando ziza kugcinwa kwinkqubo yekhompyutha. Ukunqanda nabani na ukuba akwazi ngokungqalileyo yonke idatha yakho iza kukhowudwa. Ukuqinisekisa ukuba akukho nabani na ongaphandle oya uthi afikelele kwidatha yakho, ikhompyutha iza kukhuselwa nge-password. Ngugqirha wophando kuphela oza kubanokufikelela okupheleleyo.
- Ukuba siqhagamshelana nawe ukukunika ulwazi okanye iziphumo oku kuya kubandakanya utsalo lomnxeba olunye kuphela kwinombolo enikwe nguwe.

Ndithetha nabani (okanye uqhagamshelwano) ukuba ndinayo nayiphi na imibuzo malunga nophononongo?

Ukuba unayo nayiphi na imibuzo malunga nophononongo, ungaqhagamshelana nogqirha wakho wophononongo, uGqirha Sophia Pathai okanye iSekela loPhando/uNesi (xxxxx). Xa unokuthi ufune umguquli, uza kumnikwa.

Phambi kokuba wenze isigqibo, samkela nayiphi na imibuzo onokuthi ubenayo.

Appendix 3e

Aging study – University of Cape Town

Thank you for agreeing to be part of this study. This leaflet explains what will happen when you come to Groote Schuur Hospital.

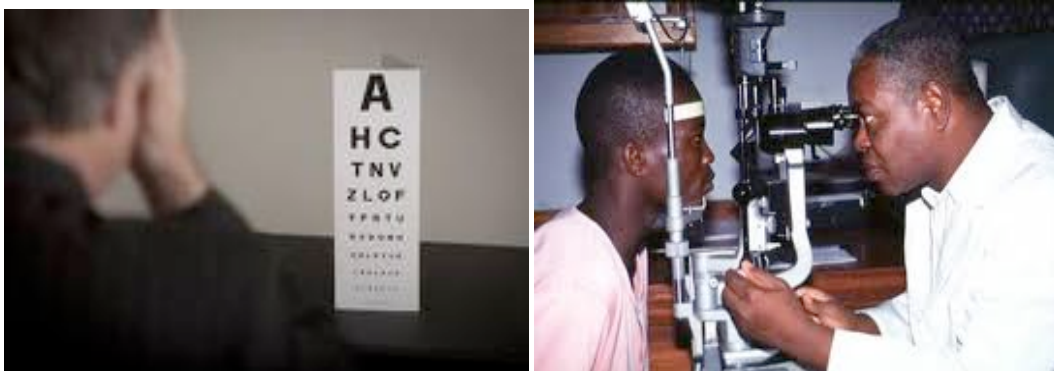
Physical tests

We will measure the strength in your arms and how fast you can walk and sit up and down.



Eye tests

We will perform some eye tests, none of them hurt. You will need to have some eye drops that make the vision blurred for a few hours but then it will go back to normal.



We will take some pictures of the eye – you cannot be identified from them.



We will also take a blood sample.

Appendix 3e

Important points:

- When you come to the hospital you will be asked to sign a 'consent form' that says you agree to take part in the study and for the information to be used in our research.
- We will not share this information with anyone else.
- You will get a complete eye examination. If you need glasses for reading you will get these too. We will also give you a total of R200 for your travel and time to attend the hospital.
- If you have an eye problem the doctor will explain this to you and will arrange for the Eye Clinic doctors to see you (at another appointment)
- We have asked for your contact details. If we call you or your friend/relative all we will mention is that we want to talk to you, not that you are taking part in study.

A more detailed information sheet is available in English and Xhosa.

Date of appointment: _____

Time: _____

If you have any questions you can contact the Study Coordinator, Tshidi on 079 337 6369

Appendix 3f

Uphononongo lobudala – eUniversity ya se Kapa

Siyabulela ukuba uvume ukuza kolopononongo. Lamaphepha axela ukuba kuzakwenzeka ntoni xa ofika esibhedlele iGroote Schuur Hospital.

Sizojonga ezizinto zilandelayo:

Siza kukhangela amandla omzimba wakho nokuzinza ngeemvavanyo ezinje ngokuphakama esitulweni, nokuhamba kwindledlana.



Amehlo

Amaqabaza amehlo aza kufakwa kwimehlo ngalinye, ukwandisa ukhozo lwemehlo isangqa esimyama esizikithini semehlo. Siza kuqiniseka ukuba ukuxilongwa kwamehlo akho kukhauleza yaye akukho buhlungu kangako.



lifoto ezithile zomva wamehlo akho ziza kuthatwa kusetyenzizwa ikhamera ekhethekileyo, akuzi kukwazeka ukubona ngokuthe ngqo kwiifoto



Appendix 3f

Siza kuthatha isapuli yegazi

Important points:

- Xa uza esibhedlele uza usayina ifom yeMvumo yabo baNokuba ngaba Thathi-nxaxheba kulophononongo.
- Asizuku xelela mtu ngalento.
- Amehlo akho aza kuvavanywa ngugqirha wamehlo. Ukubaa sifumanisa ukuba ufuna iiglaso zokubona kufutshane, ezi ziza kunikwa mahala. Uzaphiwa iR200.00 kwindleko zakho zokuza esibhedlele.
- Kwimeko engaqhelekanga yokuba uqaqanjelwe limehlo okanye ubone ngokumfiliba okugqithisileyo kufuneka ubuye esibhedlele samehlo apha.
- Ukuba siqhaqhelekanga nawe ukukunika ulwazi okanye iziphumo oku kuya kubandakanya utsala lomnxeba olunye kuphela kwinqanaba enikwe nguwe.

Iphephalinye linge English ne sXhosa elichaza kakuhle

Umhla wokuhlolwa: _____

Ixesha: _____

Xa unemibuzo thetha noTshidi kwinqanaba on 079 337 6369

Appendix 4

Willingness to participate form – Aging Study, UCT

I _____, agree to participate in this study.

I am willing to travel to Eye Clinic Groote Schuur Hospital to take part in the study.

I am willing to come to the hospital on _____ at _____.

My contact details are:

Cell no (1) _____

Cell no (2) _____ Contact person: _____

Other: _____ Contact person: _____

Signed: _____ Date: _____

For office use: Clinic ID: _____

Gugs new _____ Green clinic _____ Ema _____ (tick appropriate)

Current CD4 count: _____ Current VL: _____

Nadir CD4 count: _____ When: _____

Nadir VL: _____ When: _____

WHO clinical stage: 1 2 3 4 (circle appropriate)

Treatment regimen: _____ Duration: _____

Other comments:

Consent form

Does HIV accelerate the aging process? An assessment of ophthalmic, clinical and serum parameters of aging in a cohort of HIV-infected individuals in South Africa.

Consent Form for Potential Participants

1. I have read the information sheet concerning this study (or have understood the verbal explanation) and I clearly understand what will be required of me and what will happen to me if I take part in it.
2. My questions concerning this study have been answered by _____
3. I understand that at anytime I may withdraw from this study without giving a reason and without affecting my normal care and management.
4. I am aware that all the information that I give, all the findings of the clinician, and all the laboratory results will be kept secret.
5. I **do / do not** agree to results arising from my participation in the study being included.
6. I **do / do not** agree that my blood sample can be stored for future use at the end of this study.
7. I **do / do not** want my identity to be kept with my stored blood sample.
8. I **do / do not** wish to be contacted after the study is over for its results.
9. I **agree to take part in this study.**

Name (CAPITAL LETTERS) _____

Signature _____

Date _____

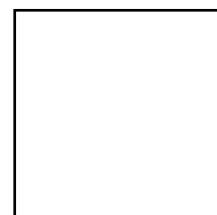
If participant is illiterate:

Name of witness

AND Thumb print of participant

Signature of witness _____

Date _____



Tick box is the participant is illiterate and refuses to have a witness present

For study staff:

I have accurately read or witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of staff _____

Signature _____

Date _____

Appendix 5b

Ifom yemvume

Ingaba i-HIV iyiqhubela phambili imo yokwaluphala? Ukuhlolwa kobunjani obunokubalwa kokwaluphala kwamehlo (ophthalmic), kokugula (clinical) nokwencindi yegazi (serum) kwiqela labantu abosuleleke yi-HIV eMzantsi Afrika.

Ifom yeMvume yabo baNokuba ngabaThathi-nxaxheba

1. Ndilifundile iphepha lolwazi ngokuphathelene nolu phononongo (okanye ndiyiqondile inkcazelo yomlomo) yaye ndikuqonda ngokucacileyo okuza kufuneka kum yaye nokuza kwenzeka kum ukuba ndithatha inxaxheba kulo.
2. Iimibuzo yam ngokuphathelene nolu phononongo iphendulwe ngu
3. Ndiyaqonda ukuba nanini na ndingarhoxa kolu phononongo ndinganikanga sizathu yaye ngaphandle kokuchaphazeleka konakekelo lwam lwesiqhelo nolawulo.
4. Ndiyaqonda ukuba lonke ulwazi endilunikayo, zonke iziphumo zikasoklinikhi (clinician), yaye zonke iziphumo zaselebhu ziza kugcinwa ziyimfihlo.
5. **Ndiyavuma / andivumi** ukuba iziphumo zam ezivela ekuthatheni kwam inxaxheba kolu phononongo zibandakanywe.
6. **Ndiyavuma / andivumi** ukuba isampuli yam yegazi igcinelwe ukuba isetyenziswe kwixesha elizayo ekupheleni kolu phononongo.
7. **Ndiyavuma / andifuni** ukuba ukwaziwa kwam kugcinwe nesampuli yam yegazi egciniweyo.
8. **Ndiyanqwenela / andinqweneli** ukuba kuqhagamshelwane nam emva kokuba uphononongo luphelile malunga neziphumo.
9. **Ndiyavuma ukuthatha inxaxheba kolu phononongo.**

Igama (OONOBUMBA ABAKHULU) _____

Usayino _____ Umhla _____

Ukuba umthathi-nxaxheba akafundanga:

Igama lengqina KUNYE Nobhontsi womthathi-nxaxheba

□

Usayino lwengqina _____

Umhla _____

Phawula ibhokisi ukuba umthathi-nxaxheba akafundanga yaye akafuni ukuba kubekho ingqina

Okwesitafu sophononongo:

Ndifunde ngokuchanekileyo okanye ndikungqinile ukufundwa okuchanekileyo kwefom yemvume kulowo unokuba ngumthathi-nxaxheba, yaye umntu lowo ulifumene ithuba lokubuza imibuzo. Ndiqinisekisa ukuba umntu lowo uyinike imvume yakhe ngokukhululekileyo.

Igama lesitafu _____

Usayino _____ Umhla _____

Appendix 6

Affix study ID label

Data entry form – Aging Study

Checked: _____
 Scanned: _____
 Database entry: _____

Demographic details

Date	<table border="1" style="width:100%; border-collapse: collapse;"> <tr> <td style="width:20%;"></td> <td style="width:20%;"></td> <td style="width:20%; text-align:center;">2011</td> </tr> <tr> <td style="text-align:center;">day</td> <td style="text-align:center;">month</td> <td style="text-align:center;">year</td> </tr> </table>			2011	day	month	year	Study ID																										
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Education	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">1</td><td>None</td></tr> <tr><td style="text-align:center;">2</td><td>Some</td></tr> <tr><td style="text-align:center;">3</td><td>Highschool</td></tr> <tr><td style="text-align:center;">4</td><td>College</td></tr> </table>	1	None	2	Some	3	Highschool	4	College	Marital status	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">1</td><td>Single</td></tr> <tr><td style="text-align:center;">2</td><td>Married</td></tr> <tr><td style="text-align:center;">3</td><td>Divorced</td></tr> <tr><td style="text-align:center;">4</td><td>Widowed</td></tr> </table>	1	Single	2	Married	3	Divorced	4	Widowed															
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1	2	3	4																															
Opportunistic Infections	<i>if CD4 and VL not avail enter '9999'</i>																																	
<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">1</td><td>Yes</td></tr> <tr><td style="text-align:center;">2</td><td>No</td></tr> </table>	1	Yes	2	No	If yes	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">1</td><td>TB</td></tr> <tr><td style="text-align:center;">2</td><td>toxo</td></tr> </table>	1	TB	2	toxo	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">3</td><td>crypto</td></tr> <tr><td style="text-align:center;">4</td><td>diarrhoea</td></tr> </table>	3	crypto	4	diarrhoea	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">5</td><td>Kaposi</td></tr> <tr><td style="text-align:center;">6</td><td>CMV</td></tr> </table>	5	Kaposi	6	CMV	<table border="1" style="width:100%; border-collapse: collapse;"> <tr><td style="width:20px; text-align:center;">7</td><td>PPE</td></tr> </table>	7	PPE											
1	Yes																																	
2	No																																	
1	TB																																	
2	toxo																																	
3	crypto																																	
4	diarrhoea																																	
5	Kaposi																																	
6	CMV																																	
7	PPE																																	

Appendix 6 Socioeconomic details

2

Checked: _____
Scanned: _____
Database entry: _____

Housing		Electricity		Toilet		Toilet	
Formal	<input type="text" value="1"/>	Yes	<input type="text" value="1"/>	In house? Yes	<input type="text" value="1"/>	Shared? Yes	<input type="text" value="1"/>
Informal	<input type="text" value="2"/>	No	<input type="text" value="2"/>	No	<input type="text" value="2"/>	No	<input type="text" value="2"/>
In home:		TV		Water tap shared?			
Stove Yes	<input type="text" value="1"/>	Yes	<input type="text" value="1"/>	Yes	<input type="text" value="1"/>		
No	<input type="text" value="2"/>	No	<input type="text" value="2"/>	No	<input type="text" value="2"/>		
Alcohol		Type		Amount per week			
Yes	<input type="text" value="1"/>	Homemade	<input type="text" value="1"/>	None	<input type="text" value="1"/>		
No	<input type="text" value="2"/>	Refined	<input type="text" value="2"/>	<500ml	<input type="text" value="2"/>		
		N/A	<input type="text" value="3"/>	500-1L	<input type="text" value="3"/>		
				1 - 2L	<input type="text" value="4"/>		
				>2L	<input type="text" value="5"/>		
Cigarettes		How many per day?		How long? _____ years		Ever smoked?	
Yes	<input type="text" value="1"/>	<input type="text"/>				Yes	<input type="text"/>
No	<input type="text" value="2"/>					No	<input type="text"/>
Drugs		What? _____ >3 times/wk?		How long? _____ years		Taken this wk?	
Yes	<input type="text" value="1"/>	<input type="text"/>		Yes	<input type="text"/>	Yes	<input type="text" value="1"/>
No	<input type="text" value="2"/>			No	<input type="text"/>	No	<input type="text" value="2"/>
				N/A	<input type="text" value="3"/>	N/A	<input type="text" value="3"/>
Snaff		>3times/wk?		How long? _____ years		Taken this wk?	
Yes	<input type="text" value="1"/>	Yes	<input type="text" value="1"/>			Yes	<input type="text" value="1"/>
No	<input type="text" value="2"/>	No	<input type="text" value="2"/>			No	<input type="text" value="2"/>
		N/A	<input type="text" value="3"/>			N/A	<input type="text" value="3"/>
Family/personal:		Where lived as a child?		Where did their mother live while pregnant with participant?			
		Rural	<input type="text" value="1"/>	Rural	<input type="text" value="1"/>		
		Periurban	<input type="text" value="2"/>	P/u	<input type="text" value="2"/>		
		Urban	<input type="text" value="3"/>	Urban	<input type="text" value="3"/>		
		Don't know	<input type="text" value="9"/>	D.K	<input type="text" value="9"/>		
Number of times pregnant		No of live births		No of breast fed babies			
<input type="text"/>		<input type="text"/>		<input type="text"/>			
N/A <input type="text" value="99"/>		(male)					
No. of partners (now and past)		Hx of STD		Yes		<input type="text" value="1"/>	
<input type="text"/>		<input type="text"/>		No		<input type="text" value="2"/>	

Physical capability measures

1. Grip Strength	*RIGHT HAND Unable	*Dominant Hand*	LEFT HAND Unable
Trial-1	<input type="text"/> kg <input type="text" value="99"/>	RIGHT <input type="text" value="1"/>	<input type="text"/> kg <input type="text" value="99"/>
Trial-2	SD <input type="text"/>	LEFT <input type="text" value="2"/>	SD <input type="text"/>
Trial-3	CV <input type="text"/>		CV <input type="text"/>
Average	<input type="text"/>		<input type="text"/>
2. Chair Rises	Unable <input type="text" value="99"/>	Time to complete 5 stands	<input type="text"/> secs If fail, no/time <input type="text"/>
3. Walking speed	Unable <input type="text" value="99"/>	trial-1	<input type="text"/> secs trial-2 <input type="text"/> secs (to 1 d.p.)
4. Walking balance	Unable <input type="text" value="99"/>	trial-1: No. of Deviations	Time <input type="text"/>
		trial-2: No. of Deviations	Time <input type="text"/>
		trial-3: No. of Deviations	Time <input type="text"/> secs
5. Balance one leg	<input type="text"/> secs		
6. Up and go test	<input type="text"/> secs		
7. Hx of eye disease	<input type="text" value="1"/> Yes	(sit walk 3m sit)	<input type="text" value="1"/> Cataract
	<input type="text" value="2"/> No	If yes	<input type="text" value="3"/> Other
		<input type="text" value="2"/> Diabetes	<input type="text" value="4"/> N/A

Appendix 6

Affix study ID label

Checked: _____

Scanned: _____

Database entry: _____

RIGHT EYE	Ophthalmic Examination		LEFT EYE								
Eye symptoms	<input type="checkbox"/> 1 Yes <input type="checkbox"/> 2 No		Eye symptoms								
If yes:	<input type="checkbox"/> 1 Dec vision <input type="checkbox"/> 2 Pain <input type="checkbox"/> 3 Floaters <input type="checkbox"/> 4 Other <input type="checkbox"/> 5 Diff near wk <input type="checkbox"/> 6 None	↓	If yes:								
Wearing gls?	<input type="checkbox"/> 1 No <input type="checkbox"/> 2 Dist only <input type="checkbox"/> 3 Near only <input type="checkbox"/> 4 Dist + Near		NEAR VISION								
DISTANCE VA	Presenting <input type="text"/> Uncorrected <input type="text"/> Pinhole <input type="text"/> Best <input type="text"/>	No of chars <input type="text"/>	BOTH EYES - presenting <input type="text"/> Chars <input type="text"/>								
VA = 20/x?	<input type="text"/>		BOTH EYES - uncorrected <input type="text"/> Chars <input type="text"/>								
	<table border="1" style="width:100%; border-collapse: collapse;"> <thead> <tr> <th>Sphere</th> <th>Cyl</th> <th>Axis</th> <th>Add</th> </tr> </thead> <tbody> <tr> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> <td><input type="text"/></td> </tr> </tbody> </table>	Sphere	Cyl	Axis	Add	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>		VA = 20/x? <input type="text"/>
Sphere	Cyl	Axis	Add								
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>								
AA (cm)	<input type="text"/>	Both eyes <input type="text"/>	<input type="text"/>								
Colour vision (x/13)	<input type="text"/>		<input type="text"/>								
Contrast sens	<input type="text"/>	Both eyes <input type="text"/>	<input type="text"/>								

Ophthalmic parameters (enter '99' if not possible)

	RIGHT	LEFT
Endothelial cell count	<input type="text"/>	<input type="text"/>
CV	<input type="text"/>	<input type="text"/>
Ex	<input type="text"/>	<input type="text"/>
CCT	<input type="text"/>	<input type="text"/>
Pentacam		
Linear	<input type="text"/> SD <input type="text"/>	<input type="text"/> SD <input type="text"/>
Peak	<input type="text"/>	<input type="text"/>
3D	<input type="text"/> SD <input type="text"/>	<input type="text"/> SD <input type="text"/>
PNS	<input type="text"/>	<input type="text"/>

Office use only:

MT	Yes <input type="checkbox"/> 1	Yes <input type="checkbox"/> 1
	No <input type="checkbox"/> 2	No <input type="checkbox"/> 2
Other parameters		
ONH	<input type="checkbox"/> 1	<input type="checkbox"/> 1
	<input type="checkbox"/> 2	<input type="checkbox"/> 2
Fundus photos	<input type="checkbox"/> 1	<input type="checkbox"/> 1
	<input type="checkbox"/> 2	<input type="checkbox"/> 2

Comments:

Appendix 6

		RIGHT EYE			LEFT EYE		
Eyelids	Normal	0			0		
	Kaposi sarcoma	1			1		
	Molluscum	2			2		
Conjunctiva	Normal	0			0		
	Pterygium	1			1		
	?SCC	2			2		
	Other	3			3		
Cornea	Clear	0			0		
	Infiltrates	1			1		
	KP	2			2		
	Other	3			3		
IOP	mmHg			9			9
<i>if patient unable to complete encircle '9'</i>							
Uveitis	Cells -grade			9			9
	Flare - grade			9			9
Post synechiae	None	0			0		
	Yes	1			1		
	Unknown	9			9		
Lens	Aphakia	0			0		
	Phakia, clear	1			1		
	Non-visually sig. cat	2			2		
	Visually sig. cat	3			3		
	Pseudophakia open capsule	4			4		
	Pseudophakia, no PCO	5			5		
	Psuedophakia +PCO	6			6		
	Unknown	9			9		
Vitreous	Clear	0			0		
	Trace	0.5			0.5		
	1+	1			1		
	2+	2			2		
	3+	3			3		
	4+	4			4		
	Unknown	9			9		
	Retina	Normal	0			0	
HIVR		1			1		
TB		2			2		
DR		3			3		
Other		4			4		
UTE		9			9		
CMVR	No CMVR	0			0		
	Unsure	1			1		
	Lesion <1/4 DA	2			2		
	Definite CMVR	3			3		
	UTE	9			9		
Macula	Nnormal	0			0		
	Atrophic	1			1		
	Exudative	2			2		
	UTE	9			9		
	RD	None	0			0	
Questionable		1			1		
Definite		2			2		
UTE		9			9		
Optic nerve		Normal	0			0	
	Pallor	1			1		
	Swelling	2			2		
	UTE	9			9		
	Valid data?	Yes	1			1	
No		2			2		

Checked: _____

Scanned: _____

Database entry: _____

Right Grade	
NS	1
CLO	2
PSC	3

Left Grade	
NS	1
CLO	2
PSC	3

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DEPT OF CLINICAL RESEARCH
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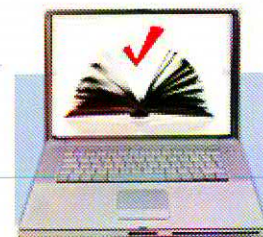
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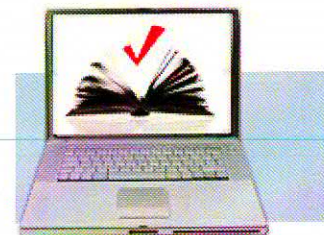
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To: Sophia Pathai [REDACTED]

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Permission is granted as long as no commercial use is made or if commercial use then UNAIDS material is a small part of the final product and proper credits are mentioned for UNAIDS and photographer in the case of a photo being used.

In the case of graphs/tables/numbers, please ensure that the latest versions are used as presented on the UNAIDS website.

Example of credit :

Credit : UNAIDS/ONUSIDA + year

For photo credits : Credits: UNAIDS/Name of photographer + year

Kind regards

UNAIDS Publications

Think Green, keep it on screen !

-----Original Message-----

From: Sophia Pathai [mailto:[REDACTED]]

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