LEUKOCYTE TELOMERE LENGTH AND LENS TRANSPARENCY AS BIOMARKERS IN POPULATION STUDIES OF HUMAN AGING

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Biomarkers of aging are indicators of characteristics of an organism which change over time. Validating aging biomarkers will enable researchers to better understand aging mechanisms and design interventions which promote healthy aging. This dissertation uses population-based cohorts to explore two emerging biomarkers of human aging, leukocyte telomere length (LTL) and lens transparency.

Short LTL records systemic oxidation and inflammation and contributes to cellular senescence. Previous studies focused on its association with diagnosed age-related chronic disease in one physiologic system and have generated equivocal results. Because disease can be undiagnosed and exist in several tissues simultaneously, previous research may have underestimated associations with LTL. We studied the association of LTL with an index of disease burden, which tabulates age-related chronic disease in five physiologic systems regardless of diagnosis. To the extent that an index across systems might capture an underlying propensity to age-related changes in all systems, a marker of fundamental aging processes such as LTL should be associated with it. We show LTL is associated with this index of disease

burden. Thus, LTL might indicate widespread incremental changes in structure or function in older adults independent of diagnosed disease.

Lens transparency may reflect systemic load of molecular glycation and denaturation, which have been associated with aging. Correlates of reduced lens transparency in humans are undefined. We studied the association of lens transparency to markers of aging and disease. We found that older adults with highly transparent lenses have longer LTL, lower prevalence of diabetes, better cognition, and lower odds of an ApoE4 allele, the strongest genetic risk factor for Alzheimer's disease. Transparency is unrelated to risk factors for atherosclerosis or non-invasively measured vascular disease.

What are the public health implications of this work? First, LTL may aid in the development of screening tools and interventions to prevent age-related disease simultaneously in multiple physiologic systems. Second, lens transparency may help detect amyloid-related brain pathology, which is vital to developing interventions to slow brain aging. Future epidemiologic research should focus on correlating changes in LTL and transparency to changes in age-related phenotypes and the ability of LTL and transparency to predict age-related outcomes.

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PREFACE

I wish to convey my sincere gratitude to the many individuals who helped me complete this dissertation. First, I am indebted to Dr. Anne Newman, my advisor and mentor for over four years. Since my arrival at the University of Pittsburgh, I never doubted her commitment to my development as a researcher and clinician. She trusted my early desire to work independently. She always knew what questions to ask to expose my deficiencies and help me learn, and when to push me to accomplish more than I predicted. Dr. Newman served as the mentor for my F30, which continues to fund my training, and provided recommendations for my successful application for other fellowships and grants. She has generously supported me to travel throughout the US and abroad, and introduced me to countless researchers to expand my network. Throughout my training she has allowed and even encouraged me to explore areas of interest unrelated to my dissertation. She is a model mentor, more than I could have asked for, and I cannot thank her enough for her investment in me.

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1.0 INTRODUCTION

Traditionally, the aging process was equated with unalterable decline and the development of disease. Although it is uncertain if a primary process of aging can be fully distinguished from the secondary process of disease, recent research has identified that aging and disease are not synonymous, and subsequently that aging can be optimized. This has been most clearly demonstrated in animal models with genetic manipulation and caloric restriction that delay aging, morbidity, and mortality. In these systems mortality rate has been the primary marker of aging, but for human studies *in vivo* markers of aging are needed. Specifically, markers of primary aging are needed as intermediate outcomes to understand the aging process and potential early benefits of preventive interventions.

This review begins by illustrating global trends in aging and priority areas for aging research, focusing on identifying and validating new biomarkers of aging. It provides a definition of a "biomarker of aging" and methodology to evaluate potential biomarkers. Next, previous research on two putative biomarkers, leukocyte telomere length and lens transparency, is described. Finally, research directions are posited to advance understanding of leukocyte telomere length and lens transparency, which in the future may be used as biomarkers to clarify the biology and physiology of aging.

2.0 BACKGROUND

2.1: What is aging, and how does aging relate to disease?

For the purposes of this dissertation I will begin with defining the terms “aging” and “disease,” which will provide guidance for considering if a marker indicates aging, disease, both, or neither. Aging is a process that has three specific characteristics. First, aging is universal – it happens to everyone, it is inescapable. It would be very difficult to confuse a 20 year old for a 40 year old or a 40 year old for a 70 year old. While disease is selective, burdening certain individuals within a population and sparing others, everyone exhibits changes with age regardless of their disease status. Second, aging is irreversible – it cannot be entirely undone. Although genetic manipulation,[1] caloric restriction,[2] and stem cell transplantation in progeroid mice[3] have demonstrated that the rate of aging can be slowed, aging cannot be completely reversed and will progress towards the ultimate endpoint of death. This is in contrast to disease, some of which can be cured or kept at bay for years or decades. Third, aging is deleterious, that is, it converts a robust organism able to respond adequately to stress into a frail organism that is susceptible to failure in the face of stress. Each individual may have their own optimal point or average setpoint for what is “robust” or “frail,” but aging will progressively impair one’s ability to respond to stress. These three characteristics – universal, irreversible, deleterious – can be used when considering if a marker measures aging – i.e., that marker occurs or exists in everyone and changes in amount, form, or purpose with age; its age-related change cannot be completely

reversed; and its age-related change ultimately hinders the individual more than it benefits the individual.

The relationship of aging to disease is somewhat unclear and can be described in several ways. These descriptions impact if one views a marker as illustrating aging, disease, both, or neither. One description posits aging is a distinct process (universal, irreversible, deleterious) that is entirely independent of the development of disease. This theory is based on the relatively consistent observation that a decline in function is seen between and within species with widely different physiology and pathology, supporting the idea that organisms share a common aging process that is independent of species-specific disease.[4] The human body undergoes many changes over time that occur without disease and are deleterious, such as thinning and wrinkling of skin and graying of hair; loss of visual accommodation, hearing sensitivity, and smell; increased blood pressure; decreased maximal heart rate, kidney function, bone mineral density, muscle mass, peripheral nerve discrimination, and thirst drive; impaired thermoregulation and immune function; intracellular accumulation of lipofuscin; increased neurofibrillary tangles and senile plaques; cross-linking of collagen; and many others.[5] These changes might be considered consequences or correlates of aging rather than disease, with the caveat that they may be highly tissue, organ, or process-specific. They may reflect aging of a single parameter of the body rather than providing insight on the aging of entire organism.

The separation of aging and disease may also be partly artificial, though, and belie our poor understanding of the aging-disease dichotomy.[6] It is not difficult to identify age-associated changes (some noted above) and consider these changes independent of disease because they do indeed occur in the absence of disease. But, it is possible that aging and disease pathogenesis are inextricable and, in the extreme, are in fact the same process expressed in different terms, at

different amounts, or at different stages along a continuum that are arbitrarily separated by humans.[6] For example, the Baltimore Longitudinal Study of Aging recruited faculty in Baltimore from across the age span and excluded people with a history of any diagnosed health condition for the express purpose of exploring physiological changes with aging rather than disease. Investigators documented that glomerular filtration rate (GFR) measured with creatinine clearance decreases on average ~1 mL/min/year. Nonetheless, over 20+ years, 30% of individuals maintained kidney function, others decreased at >2 mL/min/year, and a small number had a statistically significant increase in GFR.[7] If kidney disease is categorized using GFR cutoffs, with a GFR <60 mL/min delineating stage 1 chronic kidney disease and GFR <15 mL/min delineating end stage renal disease, when does declining GFR biologically change from being a marker of renal aging to a marker of kidney disease? In the brain, senile plaques and neurofibrillary tangles accumulate with age in the absence of clinically-diagnosed Alzheimer's disease.[8-12] Although younger patients with Alzheimer's disease have far greater densities of plaques and tangles compared to age-matched controls, the difference between cases and controls is somewhat mitigated at older ages (>80 years old). Furthermore, there is a wide range of cognitive performance among patients with similar levels of plaques and tangles, even when those levels are high.[8-12] These associations become more difficult to interpret as cognition itself is more finely divided into normal, mild cognitive impairment, and dementia. Are plaques and tangles markers of brain aging, disease, or both? As another example, bone mineral density declines with age, but with the advent of noninvasive imaging to quantify density (e.g., dual-energy X-ray absorptiometry), "normal" values have been established using age and sex specific lifetime peak values. There are now clinical conditions, osteopenia and osteoporosis, defined by comparing one's bone mineral density to these "normal" values. Is low bone density a marker of

skeletal aging or a disease? The clear distinction between aging and disease has been eroded by tools which can measure disease earlier in its course. This complicates whether measurements provide insight on the biology of aging, disease, or both.

Furthermore, although it is increasingly rare with noninvasive measurements to identify individuals who age well in many domains, there are indeed exceptional individuals who attain this status.[13] Theoretically, in contrast to these extremely healthy agers all others may be seen as "diseased." The argument to separate aging and disease is thus partly rooted in biology and measurement (juxtaposing changes that occur "with normally aging" from changes that occur "with disease"), which are more objective, and partly in semantics and interpretation (i.e., how does one partition aging and disease?), which are more subjective. Separating aging from disease may illustrate humans' long-held desire to conquer disease and then aging itself, which may necessitate conceptualizing and describing aging and disease differently, and stems from a combination of emotion and philosophy.

A final description of the aging-disease relationship concerns how aging is a risk factor for disease. Across populations, aging is one of the strongest risk factors for many diseases, particularly chronic diseases, whereas other disease risk factors are typically associated with specific diseases in specific groups of people. These observations can be explained partly by the distribution of aging (aging is experienced by everyone) versus the distribution of other disease risk factors (which are not experienced by everyone), but it is also due to the effect of aging. Time-dependent damage to molecules and tissues broadly accumulates and eventually overwhelms homeostatic processes. This occurs even in the absence of other disease risk factors due to organisms' exposure to ubiquitous damaging agents like radiation (exogenous exposure) and reactive oxygen species generated from mitochondrial energy synthesis (endogenous

exposure). Therefore, a background of time-dependent damage that we call “aging” defines the base level of risk for the development of disease, but it can also interact with other disease risk factors to augment the risk trajectory for disease. Subsequently, aging is a strong risk factor for many diseases across tissues. This relationship of aging to disease makes validating biomarkers tricky. If a biomarker is associated with age, then perhaps it is a biomarker of aging, but because age is a risk factor for disease, then is the biomarker also a biomarker of disease? If the biomarker is associated with changes which occur with age, but it is also associated with other disease risk factors, how many associations and what strength of associations imply the biomarker is more one of aging than of disease? As more accurate, specific, and diverse measurement tools are developed, answering these questions could become increasingly difficult.

Herein, for simplicity, aging will be considered a process that is universal, irreversible, and deleterious, and a marker of aging will have few to no associations with risk factors for chronic disease other than chronologic age. Disease will be considered something not universal (i.e., has identified risk factors other than chronologic age, such as lifestyle and environmental exposures), possibly irreversible (i.e., there are available preventive measures to remove risk factors or clinical treatments for overt disease), but still deleterious. If a marker is an indicator of aging, disease, both, or neither will depend upon the biology of the marker itself and the current state of knowledge of associations with that marker in human populations.

2.2: Global trends in aging

In 2007, the National Institute on Aging and US Department of State identified nine global trends in aging that characterize the changing face of world:[14]

1. Life expectancy is increasing. This raises questions about the potential for the human lifespan.
2. People aged 65 and over will soon outnumber children <5 years old for the first time in history, demonstrating an increase in the raw number of older adults.
3. The world's population ≥80 years old is projected to increase 233% between 2008 and 2040, compared with 160% for the population ≥65 and 33% for the total population of all ages. Thus, there is near exponential growth in the proportion of the population that is old compared to more linear growth for younger age groups.
4. Some populations are aging while their size declines. The joint effect of longer survival and lower birth rates has created a demographic phenomenon termed squaring of the pyramid, which describes the redistribution of a population from one with a large foundation of younger individuals and fewer older adults to one with an increasingly similar proportion of younger and older individuals.
5. Non-communicable diseases are now the major cause of death among older people in both developed and developing countries. Preventive and therapeutic interventions which target chronic disease will be increasingly important to refine and apply in both wealthy and poor nations to mitigate the medical, social, and economic effects of chronic disease.
6. Family structures are changing. As people live longer and have fewer children, older adults become a larger component of the family unit, and the benefits and burdens of caring for older family members are magnified.
7. Patterns of work and retirement are shifting. Shrinking ratios of workers to pensioners and people spending a larger portion of their lives in retirement increasingly tax existing health and pension systems.

8. Social insurance systems are evolving. Countries are evaluating the sustainability of these systems and revamping old-age security provisions.
9. New economic challenges are emerging. Population aging already has and will continue to have large effects on social entitlement programs, labor supply, and total savings.

Trends 1-4 illustrate the demographic shift occurring through time – the rising tide of older adults sweeping across the world. Trend 5 describes the accompanying boom of chronic disease which tax the health system most. Trends 6-9 demonstrate that global aging will have social and economic ramifications governments must address lest the negative consequences beginning to ripple through society continue to grow. Together, these trends clearly depict the quickening pace of aging throughout the world and the pervasive effects these changes will have on populations and individuals.

2.3: Priority areas for aging research

The Academy of Medical Sciences of the United Kingdom drafted a roadmap for aging research in the hopes that new knowledge could mitigate the detrimental effects of global aging and steer populations along a better course, often referred to as healthy aging. In their report "Rejuvenating Ageing Research," the Academy states that four broad priority areas for aging research should be:[15]

1. Developing our understanding of the basic biology of healthy aging.
2. Integrating knowledge of the processes that underpin aging and age-related diseases.
3. Measuring and understanding the determinants of healthy aging in older people at a population level.

4. Translating advances in the basic biological science of aging into effective interventions to promote healthy aging.

These priority areas match the National Institute on Aging's Strategic Plan, particularly the goal to "Improve our understanding of healthy aging and disease and disability among older adults."[16] With this guidance, researchers must ask themselves: How best can we approach these priority areas? What will enable us to develop our understanding of the biology of healthy aging and translate understanding into effective interventions? As the report suggests, identification and validation of new biomarkers of aging will in part provide answers to these questions.[15] Biomarkers are particularly important in human research because tabulating mortality, an endpoint often used in the laboratory, requires long follow-up in humans and is a crude indicator of quality of life.

2.4: Working definition of a biomarker of aging

In 1988 Baker and Sprott defined a biomarker of aging as, "a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capability at some late age than will chronological age."[17] This definition has stood the test of time yet deserves comment.[18,19] First, the authors thoughtfully mention that biomarkers should not only be considered on a one-by-one basis, but that combinations of biomarkers might be advantageous. Subsequently, researchers must consider theoretical and practical limitations on which biomarkers can be combined and how biomarkers may be combined to form a composite measurement. For example, it may be inappropriate to combine biomarkers on widely different levels of biological organization, such as expression level of a protein and difficulties in activities of daily living, because it is difficult to interpret

how these biomarkers interact. Similarly, it may be inappropriate to combine biomarkers with widely different ranges, such as a dichotomous measurement (e.g., yes/no determination), ordinal measurement (e.g., number of diagnosed chronic conditions), and a continuous measurement (e.g., gait speed).

Second, the authors include the phrase "in the absence of disease," alluding to the hotly debated issue of whether aging and disease are separable, and siding with biomarkers predicting future events independent of disease.[6] As noted in **2.1**, there are hallmarks of aging which are independent of disease and aspects of aging and age-related disease which overlap. Depending on which description of aging is applied, a biomarker of aging may be argued as an indicator of primary aging (i.e., aging completely independent from disease – a process that is universal, irreversible, and deleterious), an indicator of age-related chronic disease, or an indicator of both. Preferably, a biomarker of aging would allow monitoring of a basic aging process independent of disease. But, due to the strong tie between aging and many chronic diseases, our current imperfect ability to disentangle normal age-related changes from abnormal age-related changes from disease-related changes, and the increasing use of subclinical measurements which identify "disease" at earlier points in its course, it is possible that strong biomarkers of aging may also be biomarkers of age-related disease.

Third, Baker and Sprott write that biomarkers should be used for prediction. This is logical, though it can be explicitly added that if biomarkers were useful for prediction they might serve as points of intervention for prevention of unhealthy aging and/or disease. Fourth, the authors declare that biomarkers should predict "functional capability." Although there is no accepted definition of aging, aging can be visualized as a progressive decline in function over time, particularly the inability to restore homeostasis when stressed, so this conceptualization

seems an appropriate anchor for defining a biomarker of aging. Nonetheless, "functional capability" is an unclear term. Functional ability occurs at many levels: DNA repair enzymes maintain genomic integrity; mitosis-associated factors enable successful cell division; ion channels allow cells to communicate successfully and organs to operate properly; organ-specific function is critical to maintain homeostasis; physical and cognitive function permit an organism to navigate surroundings and respond to stress; and social function is an important capability that impacts health and wellbeing. All of these, and many more, are "functional capabilities" that change with age and that a biomarker could potentially predict. Ultimately, function is only one of many outcomes which are useful to study when evaluating putative biomarkers of aging.

Fifth, the authors assert that a biomarker should predict functional capacity better than chronologic age. This idea stems from the observation that organisms of the same species with the same chronologic age exhibit great heterogeneity in health, fitness and life expectancy,[4] and that chronologic age is therefore a suboptimal predictor of health status. It must be noted that, broadly, chronologic age remains the most robust predictor of future events, so prediction better than chronologic age is very difficult to achieve. In bench research, biomarkers may provide strong insight on the biology of aging without achieving dominance over chronologic age in statistical models derived from clinical or population studies. Furthermore, in statistical models, biomarkers may increase predictive accuracy meaningfully without being a stronger predictor than chronologic age. Subsequently, it is generally not accepted as a rule that biomarkers of aging must be stronger predictors than chronologic age in statistical models, but it is desirable.

Over time the definition posed by Baker and Sprott has been questioned by both the authors and others.[18-20] In general, two aspects of the definition are widely supported: biomarkers of aging should be correlated with age and provide insight on a biological process important to

aging. Clinical utility and superiority to chronological age for prediction have been challenged as necessary aspects of a definition due to their rigidity. Outcomes used for prediction are diverse and should not be limited to a narrow view of what constitutes "function." This definition and others should serve more as a guide than gospel and should not confine research or clinical translation.

2.5: Evaluating putative biomarkers of aging

There are several proposed criteria to evaluate putative biomarkers of aging.[17-19,21] Although there are no hard and fast requirements, these criteria, rooted in theoretical and practical considerations, are useful for assessing a putative biomarker's worth and comparing the value of one biomarker to another. These criteria can be summarized as:

1. Statistical criterion: A biomarker should be correlated with age and predict the rate of aging better than chronological age.[17,18,20,21] In other words, it should tell exactly where a person is, in regards to some measurable parameter of aging, in their total lifespan. Fulfillment of this criterion can be investigated in several ways. First, using regression coefficients in a statistical model, the magnitude of the association of the biomarker with the outcome of interest can be directly compared to the magnitude of the association of chronologic age to the outcome. Ideally, when both are standardized, the coefficient of the biomarker is larger than the coefficient of age. Second, the magnitude of the standardized coefficient of the biomarker can be compared to the magnitude of the unstandardized coefficient of chronologic age to illustrate how many years of chronologic aging is depicted by a standard difference in biomarker levels. For example, if the coefficient of a biomarker on muscle mass was

ten units per standard deviation of the biomarker and the coefficient of age on muscle mass was two units per year of age, then 1 standard deviation in the biomarker would be equivalent to 5 years of chronologic aging on muscle mass. Biomarkers with more years of chronologic aging depicted by a standard difference in biomarker level are likely stronger biomarkers of aging. Third, one can test the ability of the biomarker to attenuate the effect of age. In a statistical model this can be achieved simply: one model is built including age as a covariate, and the next model is built including age and the biomarker. The degree to which the coefficient of age is attenuated by the addition of the biomarker provides evidence for how much the effect of chronologic age is explained by the biomarker. Greater attenuation suggests a stronger biomarker of aging. Significant attenuation, or more broadly, mediation, can be formally tested using mediation regression equations.[22] If longitudinal data is available, mediation can be identified with autoregressive models or latent growth modeling depending on assumptions of the relationship between changes in the mediator, independent variable, and dependent variable. Of note, continuous measurements may provide more statistical range and variance than categorical measurements. Subsequently, biomarkers which can be measured continuously may be able to account for more variance in a given outcome, so they may be more powerful predictors on this basis alone. It is important to consider what represents the “rate of aging” when testing a biomarker’s predictive ability. A rate implies change over time, so ideally the biomarker would be used to predict a change in an outcome over years. This introduces additional statistical nuance if measurements are derived from populations because person-specific (e.g., linear regression with random intercept) or population-

average (e.g., generalized estimating equation) models can be fit to estimate the biomarker's ability to predict an outcome's change in an individual or average change for the population, and each slope is interpreted differently. More often than using a rate of change in an individual, a biomarker is used to predict a population's mortality, or rate of death, or some representation of longevity, such as high gait speed and cognitive function. Finally, when predicting an outcome, various statistical values can be used to illustrate the quality of prediction. Examples include the amount of variance in the outcome accounted for by a biomarker (e.g., using partial r^2 values) and the overall accuracy of prediction (e.g., using the area under the curve method to determine a concordance statistic).

2. Biological criterion: A biomarker should monitor a basic process that underlies aging. This basic process is likely conserved across species. Fulfillment of this criterion requires careful examination of the literature and one's own data. It is possible that species-specific biomarkers of aging exist though the commonality of aging across species implies that a core set of processes are shared, and that identifying biomarkers of these processes will provide the most insight on the biology of aging. Basic processes include those operating at the molecular, cellular, tissue, organ, organ system, and whole organism levels. These include alterations in the somatic genetic code (viral inclusions, errors of DNA replication and repair), transcriptional accessibility (epigenetic changes), transcriptional volume (mRNA content), translational volume (protein production), post-translational modification (glycation, oxidation, nitration, phosphorylation, racemerization, isomerization, ubiquitination), tissue histology and morphology (changes in tissue elasticity, lipofuscin

accumulation, plaque formation), oxidative stress, inflammation, cellular senescence, organ function, and physical and cognitive function. Because biomarker research advances with the goal of translating biomarkers to the clinic for diagnosis, monitoring therapy, or treatment, knowing which basic process a biomarker monitors as a passive indicator or active mechanistic contributor also helps ensure informed translation to minimize unforeseen negative consequences.

3. Clinical criterion: A biomarker should be able to be tested repeatedly and accurately without harming the person. Ideally, it is also measured inexpensively, efficiently, and reproducibly. Fulfillment of this criterion maximizes the usefulness of the biomarker as a potential clinical tool. Nonetheless, if a biomarker provides meaningful data but its measurement is impractical, investigators should not dismiss it outright because future innovations may allow easy, cheap, safe, and accurate measurement.
4. Experimental criterion: A biomarker should act and be measurable in both humans and laboratory animals so that it can be tested in animals before being validated in humans. Observational and experimental studies in model systems are often simpler, cheaper, and quicker than similar studies in humans, and can be used to vet biomarkers in a semi-high-throughput fashion before expending resources on human studies. Even complex biomarkers, such as indicators of mobility and activity, have been measured in lower order animals like *C. elegans* and rodents. Although some biomarkers will be specific to humans or act differently in humans, validation across species can uncover fundamental mechanisms underlying aging and disease.

In addition to these criteria, it is important to note what outcomes are used to evaluate biomarkers, as described in **2.1**. For example, decline in pulmonary function is universal, irreversible, and deleterious, and thus it would appear valid to test a biomarker's ability to predict pulmonary function if the biomarker were being validated as a biomarker of aging. In contrast, it would likely not be appropriate to use chronic obstructive pulmonary disease (COPD) as an outcome, because this represents a disease phenotype rather than an aging phenotype, and mechanisms leading to an age-related decline in pulmonary function, such as stiffening of the airways and alveoli and decreased thoracic muscle strength, may be quite different from mechanisms leading to COPD, such as smoking. Furthermore, assessment of biomarkers is complicated by the fact that biomarker can serve as both a predictor and an outcome: in the previous example pulmonary function was used as an outcome, but it could also be used as a potential biomarker of aging because it fulfills the above criteria, and thus it could serve as a predictor of an aging-related outcome such as incident disability. (Indeed, in the following papers, leukocyte telomere length, a putative biomarker of aging, will be used as both an outcome (**4.0: Paper 1**) and a predictor (**5.0: Paper 2**)).

2.6: Examples of putative biomarker of aging

Many biomarkers of aging have been posited; examples are listed in Table 2.9.1. They range from alterations in the genome (e.g., DNA methylation) to whole-body function (e.g., gait speed). In general, biomarkers at lower levels of organization (genetic, molecular) have been examined in human populations less often than biomarkers at higher levels of organization (tissue, organ) because of the novelty of high-throughput genetic and molecular assays. Study of many tissue and organ-based biomarkers, such as aortic calcification and pulmonary capacity,

have been driven by their strong associations with widespread environmental exposures (e.g., smoking) and burdensome chronic diseases (e.g., coronary artery disease, COPD). This again illustrates the dichotomy between aging and disease.

In vivo measurement of pathology in some tissues, such as white matter hyperintensities in the brain, remains relatively difficult or expensive on a population level, but function can be measured simply and cost-effectively (e.g., Digit Symbol Substitution Test) and serve as a valuable adjunct. Although some biomarkers can be categorized easily in one organizational level (e.g., activities of daily living), other biomarkers may span several organizational levels. For example, serum glucose and lipids (not listed in the table) are molecules, though they could be classified as markers of tissue function including the small intestine, pancreas, liver, skeletal muscle, fat stores, and even brain. Furthermore, biomarkers frequently reflect several processes which may contribute to aging rather than a single process. Investigators must pay close attention to the sensitivity and specificity of biomarkers to illustrate particular processes and/or levels of organization and acknowledge lack of specificity and sensitivity when appropriate.

In the following pages, two putative biomarkers of aging, telomere length and lens transparency, will be examined using the criteria described previously. This provides a basis for future studies to investigate their value as biomarkers of aging. Telomere length and lens transparency were selected for investigation for several reasons. First, both are relatively novel: lens transparency has been uninvestigated in population-based studies as a biomarker of aging, and although telomere length has been studied for a decade in epidemiologic research, many questions regarding its validity remain unanswered. Thus, future studies will be valuable to the scientific community. Second, these biomarkers are measured in different tissues and may reflect different processes which contribute to aging. Telomeres are part of DNA and telomere length is

chiefly measured in peripheral mononuclear lymphocytes using molecular assays. Telomere length is believed to integrate measurement of systemic oxidation, inflammation, and cellular senescence, and may reflect the aging immune system and/or the immune system's interaction with the rest of the body, particularly the vasculature. Transparency is a physical property of the lens that is a function of protein integrity and is measured using optical devices. It can be measured repeatedly as an *in vivo* tissue-specific marker, but the proteins that comprise the lens are used throughout the body for the same purpose: to maintain protein conformation, especially under stress. Few direct markers of the maintenance of protein integrity are available to use in epidemiologic research, so lens transparency might be particularly valuable for measuring this key aspect of aging.

2.7: Telomere length as a biomarker of aging

Telomeres are repeating TTAGGG nucleoprotein caps flanking nuclear DNA. With replication of nuclear DNA during mitosis, telomere length progressively shortens because the replication machinery cannot copy the absolute ends of DNA, termed the end replication problem. With critically short telomeres, the cell exits the cell cycle and becomes senescent.[23,24] Importantly, this process protects against unbridled cellular division, which can lead to cancer.

Telomere length has been postulated as a marker and/or fundamental regulator of cellular and thus organismal aging.[25,26] The following sections summarize the literature on telomere length as a biomarker of aging, particularly with respect to aforementioned criteria for evaluating a putative biomarker of aging: 1) monitoring a basic process that underlies aging; 2) predicting the rate of aging; 3) able to be tested repeatedly and accurately without harming the person; 4) acting in both humans and laboratory animals.

2.7.1: Telomere length monitoring a basic aging process

Currently, determinants of telomere length are poorly understood, and thus it is unclear what biologic processes telomere length may reflect. At birth telomere length is highly heterogeneous, ranging from roughly 5,000-15,000 basepairs. Linkage analyses and genome wide association studies have identified few possible loci influencing LTL, most notably those close to *TERC,* which appears mechanistically plausible because it encodes the template RNA component of telomerase, the enzyme which elongates telomeres.[27-31] Previous reports show LTL is heritable[27,28,32-34] and modified by paternal age at conception.[35-38]

Of note are hereditary diseases caused by mutations in the telomerase complex. (Telomere length can also be lengthened by homologous recombination during mitosis and chromosomal end joining, though the latter introduces genomic instability.) Patients with these diseases, including dyskeratosis congenita (DKC), bone marrow failure syndromes (BMFS), and idiopathic pulmonary fibrosis (IPF), may be considered to suffer from premature aging because they all display chromosomal instability and accelerated cellular senescence, particularly in tissues which proliferate frequently.[39] The clinical characteristics most common to these three conditions are compartment-specific or complete bone marrow failure, which could be seen as a more extreme form of age-associated senescence of hematologic progenitor cells, and cancer, which is also age-associated. Although some mutations, such as those in TERC and hTR, are associated with all of these diseases, presentation is nonetheless distinct. For example, the age of onset for DKC is before age 60 (typically between ages 10-30), for BMFS is all ages, and for IPF is usually after age 40. Short telomeres appear in all DKC patients, some BMFS patients, and in an unknown proportion of IPF patients. Other aspects of these diseases, such as pulmonary

fibrosis, nail dystrophy, and leukoplakia, are not seen with normal aging. Subsequently, these diseases do not exactly mirror normal aging, so it is difficult to use them as models for a potential impact of short telomeres on aging in the general population.

Werner's syndrome provides stronger evidence for a mechanistic role for telomere length in aging. Werner's syndrome is characterized by a mutation in the *WRN* gene that encodes a DNA helicase and exonuclease important for DNA replication, repair, and telomere maintenance. Patients have short telomeres and display normal development until lack of a pubertal growth spurt, which is accompanied by hypogonadism, short stature, flat feet, cataracts (bilateral in nearly 100% of patients), dermatologic pathology, graying/thinning hair, type II diabetes, osteoporosis, soft tissue calcification, premature atherosclerosis, and cancer.[40] By their 30s and 40s patients look like they are several decades older. In general, Werner's syndrome patients display a phenotype that appears more like accelerated aging compared to patients with DKC, BMFS, and IPF. Nonetheless, Werner's syndrome is not entirely comparable to normal aging. In particular, the dermatologic pathology (tight skin, hyperkeratosis, ulceration) and types of cancer (mesenchymal cancers such as sarcoma, multiple synchronous cancers, and rare cancers) experienced by Werner's patients are not similar to the dermatologic changes (skin wrinkling, sagging, and thinning) and cancers (predominantly epithelial of the colon, prostate, and female reproductive organs) seen with normal aging. Subsequently, Werner's syndrome suggests but does not prove that telomere length plays a role in normal aging.

What biological processes, then, might telomere length reflect? There is a great deal of *in vitro* and *in vivo* evidence that telomere length is affected by two processes in particular: cellular replication and oxidation. Regarding replication, as stated previously, the DNA replication machinery is unable to copy the extreme ends of chromosomes during mitosis. Thus, telomeric

DNA is progressively lost with each cell division. It is estimated that roughly 50 base pairs are lost due to the end replication problem.[41] Most age-associated shortening occurs during rapid somatic expansion, i.e., growth from birth through puberty.[25,42] With critically short telomeres the cell exits the cell cycle and becomes senescent.[23,24] Subsequently, telomere length may reflect the growth rate or remaining replicative potential of a population of cells.

Telomere length also shortens with increasing oxidative stress.[25,43] In fact, in human diploid fibroblasts cultured under normal conditions, the major determinant of telomere shortening appears to be single stranded breaks in DNA caused by oxidative stress.[44] The large effect of oxidation on telomeric DNA occurs because of a specific deficiency in base excision repair.[44,45] Single stranded breaks result in telomere shortening during replication,[46] most likely due to temporary stalling of the replication machinery.[47] *In vitro*, oxidative stress increases the rate of telomere shortening by an order of magnitude.[44,48,49] Reduction of oxidative stress decreases the rate of telomere shortening and postpones replicative senescence.[46] Depending on where the single stranded break occurs, the lesion can cause either no effect on telomere length or up to a 1000 base pair loss of telomeric DNA.[41] Modeling studies that predict oxidative stress is the major contributor to telomere shortening match experimental results.[41,43]

In sum, evidence suggests that, of basic biological processes, telomere length most likely reflects somatic growth before the end of puberty and cellular senescence and oxidative stress after puberty. Most other evidence is derived from correlation in general population samples (detailed in **2.7.2**). Subsequently, it is unknown what biological pathways telomere length may indicate about aging beyond cellular senescence and oxidative stress. It is possible that short telomeres induce senescence, which upregulates secretion of inflammatory factors that promote aging. In this case short telomere length may be a cause of inflammation and aging more than a

consequence of these processes. Furthermore, it is unknown if accelerated telomere shortening is a cause of accelerated aging in the general population because data on phenotypic associations with short telomeres are derived from clinical studies of patients with distinct genetic syndromes.

2.7.2: Association of telomere length with age, sex, race, markers of health status, inflammation, age-related chronic disease, function, and mortality

Leukocyte telomere length (LTL) has been studied in association with many phenotypes in cross-sectional epidemiologic studies. Major findings from studies with at least several hundred participants are listed in Table 2.9.2. If LTL was truly associated with a phenotype one would ideally observe consistency across populations, measurement methods, and statistical models. This consistency has only been observed for associations with age, gender, and race. Shorter LTL is associated with older age, male gender,[33,50-61] and Caucasians race.[33,59,62] The strength of the association with age is highly dependent on the age range of the population. Male gender and Caucasian race appear to be associated with a mean LTL that is several hundred basepairs shorter relative to LTL in women and African-Americans or Hispanics, respectively. The causes of these large gender and race effects are unknown.

Associations between LTL and other markers of health status are in general equivocal and do not appear dependent on differences between study populations, measurement method, or statistical adjustment. These include sociobehavioral factors (smoking,[50,51,57-59,63,64] alcohol consumption,[50,51,65] physical activity,[51,55,59,60] socioeconomic status[66,67]), body mass index,[33,50,51,57-59,63,64,68] lipids,[51,58,62,68] markers of glucose metabolism,[51,56-58,60,68] and blood pressure.[51,52,57,58,68] LTL has been inconsistently associated with markers of subclinical cardiovascular disease including carotid or femoral intima-media thickness,[53,57,63,69] ankle-

brachial index,[57] coronary artery calcium,[70] and pulse wave velocity.[52] Despite these discrepancies, some have argued that there is a robust association with atherosclerosis[33,51,53,54,56,57,63,71-75] because there are plausible mechanistic reasons for an association between shorter LTL and atherosclerosis.[76] In particular, with inflammation there is increased demand for hematopoetic cells that propagates up the lineage chain until it includes hematopoetic stem cells. Increased stem cell division leads to progressively shorter telomeres in both stem cells and differentiated cells that continue to divide in the periphery (e.g. lymphocytes). In total, the hematopoetic pool becomes "aged" as the population of cells edges closer to senescence. Subsequently, with age and inflammation there would be less division of circulating hematopoetically-derived cells and epithelial progenitor cells, which would then be less able to maintain the vascular wall in response to oxidative stress and plaque growth.[76] Alternatively, because oxidative stress and inflammation are associated with atherosclerosis, and there are consistent associations between markers of oxidation and inflammation (e.g., interleukin-6, C-reactive protein, homocysteine, isoprostane urinary 8-epi-PGF2α) and shorter LTL,[51,56,57,60,77] atherosclerosis and shorter LTL may be non-causally linked via these shared mechanisms.

Pulmonary function[65,78] and bone mineral density,[60,61,79] markers of specific tissues which decline with age independent of disease, are likely not or only weakly associated with LTL. Grip strength, a marker of physical function, has not been associated with LTL.[65,78,80] An association between LTL and cognitive function is equivocal. In 382 women not diagnosed with dementia or cognitive impairment, shorter LTL was independently associated with worse memory and learning,[81] which was corroborated by data from the Nurses' Health Study[82] and a hospital-based case-control study,[83] but not by a cohort of 449 inpatients in which there was no difference in

LTL between patients who were cognitively normal, had mild cognitive impairment, or were demented.[84] A report from the Health Aging and Body Composition Study, where LTL was examined in association with cognitive function in 2,734 non-demented community dwelling older adults, provides inconclusive results.[85] Furthermore, LTL is likely not associated with ApoE genotype, the strongest genetic risk factor for Alzheimer's disease.[85,86]

Telomere length has been used in several epidemiologic studies as a predictor of lifespan or death. Cawthon first reported an association between shorter LTL and increased mortality in individuals aged 60 or older.[87] In this study, increased mortality was specifically due to higher rates of cardiovascular disease and infectious disease-specific death. Since that report was published, some other studies have found that shorter LTL is associated with increased mortality[88-92] and others have not.[57,65,93-95] Of note, Epel et al. found that shorter LTL was only associated with CVD-specific mortality in women and not men, and not with overall mortality.[90] In most of these studies, models have been minimally adjusted, typically only for age, and occasionally for sex or race. Differences in measurement methods for LTL (Southern blot vs. qPCR) may contribute to inconsistency between studies (see **2.7.3**). Furthermore, with increasing age, the variability in LTL in a population may decrease.[93,94] Because these studies were conducted in individuals who were on average older than 60 years, it is possible that they had reduced power to detect associations between LTL and mortality. In sum, LTL appears weakly associated with overall mortality, and possibly more strongly with CVD or infectious-disease specific mortality.

Finally, several longitudinal studies have been conducted to determine predictors of change in LTL over time.[71,90,91,96-99] In these studies, time between samples of LTL ranged from 2.5 to 10 years. All except one[71] relied on qPCR to measure LTL. All found that baseline LTL

was the strongest, and often the only, predictor of the subsequent LTL measurement, and several noted that 11-34% of the population increased in LTL during the time period.[71,91,96,98,99] In patients with stable coronary artery disease, Farzaneh-Far et al. found that higher omega-3 fatty acid levels were associated with less decline in LTL,[98] though this has not been corroborated. Although studies of changes in LTL have the potential to provide the strongest evidence for what LTL may reflect in humans, those published to date have substantial methodologic flaws.[100,101] In particular, the time between telomere measurements was short, which resulted in small changes in mean LTL during the follow-up period. If telomere attrition in late life is approximately 30-100 basepairs per year,[100] five years of change would result in only 150-500 basepairs of shortening, which was generally observed in these longitudinal studies. Given that inter-individual variation in LTL is approximately 5,000-10,000 basepairs (depending on mean age of the population) and most longitudinal studies of LTL change have used quantitative polymerase chain reaction for measurement, which has a higher coefficient of variation (see **2.6.3.2**), and have had a small sample size, it is highly likely that these studies were underpowered to detect possible associations between change in LTL and outcomes given the degree of measurement variability. In fact, it is possible that the changes in LTL observed during the follow-up periods were partly or mostly artifacts from measurement error. Longitudinal studies with much longer follow-up time and larger sample size are necessary to convincingly determine predictors of change in LTL and what change in LTL might predict.

2.7.3: Measurement of telomere length

Measurement of telomere length requires nucleated cells. In laboratory investigations of non-humans, any tissue with nucleated cells can theoretically be sampled with tissue sampling or

sacrifice of the research animal. For human studies, though, cells must be sampled with minimal harm to study participants. Typically, this entails a blood draw and isolation of white blood cells. Thus, telomere length in population-based human studies is almost exclusively measured as leukocyte telomere length. Subsequently, obtaining samples for telomere length is relatively harmless. It should also be acknowledged that although there is synchrony in telomere length among hematopoetic cells,[102] LTL may not be a perfect surrogate for telomere length in other tissues.

There are several platforms for measuring LTL. Measurement reproducibility and accuracy is platform-dependent. The original technique, Southern blot,[103] remains the gold standard. Cawthon introduced an assay using quantitative polymerase chain reaction (qPCR)[104] and recently an updated multiplexed version with higher throughput.[105] The third notable method involves coupling individual cell sorting using flow cytometry and metaphase DNA staining using fluorescent *in situ* hybridization (Flow-FISH),[106] which was modified into a high throughput quantitative FISH (HT Q-FISH) technique.[107] Single telomere length analysis (STELA) was developed to measure the length of telomeres on single chromosomal arms.[108] Each platform has different strengths and weaknesses and measures a different part of the telomere complex. Furthermore, measurement error is introduced in multiple places (e.g., within and between gel error for Southern blot; within and between wells and plates error for qPCR), but this error is rarely incorporate into statistical analysis. Because of heterogeneity in results from population-based studies of LTL, there is considerable debate over how measurement platform may impact findings and weaken comparability of results across platforms.[26] Also of note is that Southern blot and qPCR, the methods chiefly used in epidemiology, return mean LTL. It is unknown if cellular senescence is preferentially induced in the presence of one

critically short telomere or shorter average telomere length. Thus, measurements from these platforms may not capture the biology of telomere length important to aging.

2.7.3.1: Southern blot

The Southern blot technique for measuring LTL was developed by Aviv and colleagues chiefly to examine LTL in epidemiologic studies.[103] Nonetheless, it can be modified to measure telomere length in any nucleated cell type with intact DNA if telomeres are within the detectable limits of the technique. "After extraction, DNA is inspected for integrity, digested, resolved by gel electrophoresis, transferred to a membrane, hybridized with labeled probes and exposed to X-ray film using chemiluminescence."[103] The protocol notes that a skilled technician could process approximately 130 samples per week.

Strengths of Southern blot include its reproducibility (coefficient of variance typically <2%) and expression of values in absolute base pair units (though measurements are terminal restriction fragments due to the use of restriction enzymes to digest the DNA). It also allows determination of telomere length distribution, which can be used as a parameter of interest besides mean LTL, though seldom is because it requires accurate measurement of the width of the gel bands relative to reference DNA, which is difficult. Weaknesses of Southern blot include requiring a considerable amount of DNA (3 μg per sample) and measuring both the telomeric and subtelomeric regions. Inclusion of the subtelomeric region may result in artificial inflation of the mean LTL. Cawthon has commented that differences in the subtelomeric region can also cause variation in terminal restriction fragment lengths when different restriction enzymes are used,[104] which has been observed with direct comparison of results following digestion with *Hin*fI/*Rsa*I, the most common restriction enzyme used for Southern blot, vs. *Hph*I/*Mnl*I.[33]

2.7.3.2: Quantitative polymerase chain reaction

Cawthon's original qPCR assay was created to provide a high throughput technique to measure LTL.[104] This method measures the relative average LTL in genomic DNA by determining the ratio of telomere repeat copy number to single copy gene copy number (T/S ratio) in experimental samples relative to a reference sample. The mean LTL in base pairs is calculated by multiplying the T/S ratio by the length of the reference gene. In the original description of the assay, the overall coefficient of variance was 5.8% and the linear correlation between T/S ratio and terminal restriction fragment length was 0.677 ($P=1.5x10^{-24}$). Cawthon stated that average LTL differing in as little as 11.4% could be resolved using this technique at 95% confidence.[104] Recently, Cawthon introduced a multiplex version of the qPCR assay to eliminate variance in T/S ratio introduced by pipetting different quantities of DNA into separate wells, and reduce cost and increase efficiency by requiring half the number of reactions.[105] T/S ratios obtained via the multiplexed assay are more highly correlated with terminal restriction fragment lengths ($R^2=0.844$). The intra-assay coefficient of variance is 5.22%, and the inter-assay coefficient of variance is 3.13%.[105]

PCR has the advantages of requiring far less DNA (50 ng per sample) and achieving true high throughput performance. In general, the variance of qPCR is believed to be >2%, and in the literature is often quoted as 5%-10%. Because LTL is highly heterogeneous between individuals of the same age, and rates of LTL shortening vary by an unknown amount, this higher variance can weaken the ability to resolve significant differences between mean LTLs.[103] For example, in cross-sectional studies of LTL, the observed difference in mean LTL between case and control groups is commonly several dozen to several hundred basepairs.[100,103] With an assay coefficient

of variance of 2%, individuals with a baseline LTL of 5,000-15,000 basepairs could register a difference in LTL of 100-300 basepairs simply due to measurement error, which could easily obscure observed differences in mean LTL between cases and controls. Furthermore, stability of the reference gene is not guaranteed, and there is no agreed-upon reference gene despite frequent use of albumin or beta-globin.[103-105] For these reasons, qPCR has been questioned as an accurate technique for telomere epidemiology despite having higher throughput and lower cost.

2.7.3.3: Flow-FISH and STELA

Lansdorp developed the original Flow-FISH technique to allow measurement of mean telomere length with metaphase staining from any subpopulation of circulating peripheral cells isolated using flow cytometry as a first step.[106] Subsequently, Flow-FISH has the distinct advantages of returning telomere length for individual cells of distinct cell type and the distribution of telomere lengths of that cell type. Because internal controls and highly specific nucleic acid probes are used to hybridize to the TTAGGG telomeric repeats, Flow-FISH is more accurate than other methods which are prone to including sub-telomeric sequences (Southern blot), influencing telomere length depending on the restriction enzyme used (Southern blot), missing some sequence due to less-specific probes (qPCR), or relying on stability of a reference gene to measure relative LTL (qPCR). Human error is reduced by automation of most pipetting and cell sorting, and systemic error can be minimized by inclusion of internal controls with known telomere lengths. The original protocol suggests that telomere lengths from 22 different individuals can be measured in 12 hours over 2-3 days. Despite returning more information than other platforms, Flow-FISH is much more expensive, far less efficient, and requires greater technical expertise, limiting its use in population-based research.

HT Q-FISH was developed by Canela et al. to maintain the accuracy and flexibility of Flow-FISH while capitalizing on the efficiency achieved by plate-based technologies (e.g., qPCR), and to allow measurement of telomere length from any cell type that can be grown under conventional tissue culture conditions.[107] HT Q-FISH can analyze 96 samples with at least 1,000 nuclei in 2 hours. Because time is greatly reduced and more samples can be analyzed simultaneously, there is a reduction in error from variation in metaphase staining conditions over time and across samples.[107] The technique reportedly costs less than traditional Flow-FISH. Thus far, HT Q-FISH has not been widely employed in epidemiologic studies.

Baird et al. described STELA as a viable platform for measuring telomere length in 2003.[108] STELA was developed to provide the highest level of granularity possible when measuring telomere length, i.e. measuring telomere length for individual chromosomes rather than averaging telomere lengths across all peripheral leukocytes, subpopulations of peripheral cells, or across chromosomes in a single cell. Subsequently, STELA is a powerful tool to examine telomere dynamics. For example, it can be used to investigate how inter-individual or even inter-chromosomal differences in the genetic code (e.g., single nucleotide polymorphisms), genetic structure (e.g., proximity of binding proteins), or genetic machinery (e.g., fidelity of replication and repair complexes) may impact telomere length. Because of its high granularity and very low throughput, STELA is more amenable to basic studies of telomere biology and has not been used in epidemiologic studies.

2.7.4: Telomere length in laboratory animals

Although telomeres appear in all eukaryotes, telomere length varies widely among eukaryotic organisms. This impacts how useful particular model systems are for studying telomere length as

it relates to human aging. Yeast have telomeres that are as short as 300 basepairs.[109] Subsequently, yeast are more amenable to studies of telomere structure and dynamics. Telomeres in inbred mice are much longer (20,000-150,000 basepairs) than telomeres in humans and mice have a higher baseline expression of telomerase.[23,110] In terms of aging in mice compared to aging in humans, mice also have a higher rate of somatic expansion and shorter lifespan. But, mice are the most flexible model system to study aging in vertebrates. Subsequently, to use mice to study telomere length as it might relate to humans, the telomerase-deficient mouse was created through knockout of the murine *TERC* gene.[111] These mice have markedly shorter telomere length than typical inbred mice and their telomeres degrade at a much faster rate. Numerous studies in *Terc*$^{-/-}$ mice have demonstrated that they have a much greater rate of chromosomal end-to-end fusions, limited viability after several generations, male and female infertility, embryonic mortality due to defective closure of the neural tube, small size and severe intestinal atrophy, spleen atrophy and reduced proliferation of B and T lymphocytes, impaired germinal center function, reduced angiogenic potential, reduced proliferation of bone marrow derived stem cells, heart dysfunction, and reduced proliferation of neural stem cells.[110] These mice also display a lower incidence of cancer. Rescue with addition of telomerase (comparison to *Terc*$^{+/-}$ mice) restores homozygous knockout mice to a relatively normal phenotype with far less chromosomal instability.[112] The large effect telomere length may have on longevity was recently demonstrated when researchers knocked in telomerase activity to aged telomerase-deficient mice with short telomeres.[113] Before knock-in these mice exhibited very short telomeres and marked age-related degeneration across tissues. Knock-in of telomerase lengthened telomeres, reduced DNA damage signaling and associated cellular checkpoint responses, allowed resumption of proliferation in quiescent cultures, and eliminated degenerative phenotypes across multiple

organs including testes, spleens, and intestines. Somatic telomerase reactivation reversed neurodegeneration with restoration of proliferating Sox2(+) neural progenitors, Dcx(+) newborn neurons, and Olig2(+) oligodendrocyte populations. These studies provide strong evidence that a minimum telomere length is necessary for maintenance of viability and proper development in vertebrates. Nonetheless, they belie the difficulty in using model organisms to study telomere length because the telomerase-deficient mouse displays phenotypic characteristics that are far from normal mouse or human aging.

2.7.5: Summary of previous findings on telomere length as a biomarker of aging

Data derived from *in vitro* and *in vivo* studies strongly suggests telomere length reflects cellular senescence and oxidative stress. Data from epidemiologic and clinical studies is less conclusive. Although shorter LTL appears associated with older age, male gender, and white race, LTL has been inconsistently or not associated with other characteristics of aging or age-related chronic disease. Inconsistency may be partly due to differences between study populations, measurement methods, and statistical modeling, though it may also imply an association does not exist. Animal studies have provided important data on telomere dynamics and the impact of a minimum telomere length on organismal health, but because telomere length is highly species-specific and alterations in model systems do not appear like normal aging, it is difficult to extrapolate conclusions from lower order animals to humans. Currently, it remains inconclusive if telomere length is a biomarker of aging for a whole organism or a biomarker of aging in specific tissues in human populations.

2.8: The lens

The lens sits in the anterior chamber of the eye behind the cornea, aqueous humor, and iris, and in front of the vitreous humor. It is suspended by the ciliary zonules, which in turn pull on the lens to alter its conformation, allowing the lens to properly focus light on the retina. The lens is encased in a thin capsule. The lens itself has a central core, or nucleus, which is surrounded by the lens cortex. Thus, the structure has been likened to a candy peanut M&M, with the lens capsule represented by the thin candy coating encompassing the lens, the lens cortex represented by the chocolate covering, and the central lens nucleus represented by the peanut. The lens is roughly circular, convex when viewed from outside the body, and has distinct anterior and posterior faces.

The lens is unique compared to other human tissues in that nuclear lens cells (primary fiber cells) are present at birth and remain essentially unchanged throughout life, so its contents are not altered by turnover or removal processes. The lens grows in thickness throughout life by cells progressively layering on the nucleus and building the cortex (secondary fiber cells), similar to the growth and appearance of an onion. More specifically, cells differentiate and migrate from the anterior lens epithelium to the rostral and caudal tips of the cortex, the lens poles. As the cells migrate to and turn at the poles, reaching a line termed the equator, they become secondary fiber cells, eventually flattening, lengthening, and stacking on each other. Throughout differentiation, migration, and flattening, the secondary fiber cells produce large amounts of lens crystallin, the natively clear protein that eventually comprises more than 90% of the protein mass of the lens, 50% being alpha-crystallin.[114] Simultaneously, secondary fiber cells degrade all their organelles, which if remaining would scatter light and render the lens useless. The end result is a core of

primary fiber cells surrounded by layers of secondary fiber cells that are cell membranes full of crystallin protein and very little else, including no organelles.

2.8.1: Lens biology and associations with aging in model organisms and humans

The biology of the human lens makes lens transparency a particularly attractive candidate biomarker of aging. Lens transparency decreases when crystallins, which are normally clear and water-soluble, undergo oxidation, glycation, and other biochemical changes that alter their conformation, causing them to aggregate, precipitate, and scatter light.[114,115] Alpha-crystallin is composed of alpha-A (non-stress inducible) and alpha-B (stress inducible) crystallins, which both function as chaperones, proteins critical to maintaining cellular integrity by preserving protein conformation, especially under stress.[114,116,117] Lens epithelial cells induced to overexpress alpha-A or alpha-B crystallin have enhanced resistance to thermal, photochemical, and other stress conditions.[118,119] In contrast, knockout of the alpha-A crystallin gene causes higher levels of lens epithelium cell death, even without stress.[120] Because lens crystallins exist in a relatively homogenous environment surrounded by other lens crystallins, they function to maintain the conformation of each other. Lens proteins that aggregate and precipitate reduce transparency directly but also indirectly by not being able to maintain the conformation of adjacent crystallins, which may in turn aggregate and precipitate. Lens transparency thus reflects the integrity of the crystallins and the ability of the lens to maintain protein conformation.

In addition to the lens, alpha-A crystallin is found in embryonic tissue, pituitary gland, placenta, and spleen, while alpha-B crystallin is found in brain, kidney, heart, extraembryonic tissue, mammary gland, liver, muscle, testis, thymus, lung, pancreas, thyroid, skin, uterus, ovary, colon, diaphragm, limb, and placenta.[121] In these tissues crystallin also acts as a chaperone.

Eerily, alpha-crystallin dysfunction or absence mimics an aging phenotype, particularly in skeletal muscle and brain. In alpha-B crystallin-knockout mice, there is marked and accelerated skeletal muscle degeneration (sarcopenia) and kyphosis.[122] This is supported by other studies showing that alpha-B crystallin links the differentiation and apoptosis-resistance programs in myocytes, prevents apoptosis, and maintains the tubulin cytoskeleton in skeletal muscle cells.[123-125] In brain, alpha-B crystallin is present in limbic and paralimbic regions, which are commonly affected in Alzheimer's patients, and the number of alpha-B crystallin positive neurons increases in parallel with neuronal loss.[126] Alpha-B crystallin is upregulated in astrocytes associated with senile plaques and cerebral amyloid angiopathy in Alzheimer's patients,[127] and it inhibits aggregation of A-beta peptide *in vitro*.[128] In summary, alpha-crystallins are intimately and ubiquitously involved in cellular maintenance that is necessary for survival with aging. Depletion of or defects in alpha-crystallin is associated with a range of age-related pathologies, while elevation of alpha-crystallin appears protective. Because alpha-crystallin is most prevalent and homogenous in the human lens, lens transparency might sensitively reflect the biologic integrity of a human during the lifespan.

Strong evidence that the lens may reflect aging could come from studies of caloric restriction, which extends lifespan in several species.[4] Unfortunately, although caloric restriction studies have begun in humans, restriction is usually short term, and the lens has not been examined in these studies. Nonetheless, phenotypic changes have been observed in the mouse and rat lens with caloric restriction. In several strains of mice and rats that display lifespan extension with caloric restriction, *in vitro* and *in vivo* analysis demonstrate restriction reduces onset of age-related cataract, slows decline in age-related proliferative capacity of lens epithelium, and reduces age-related lens epithelial telomere shortening.[129-134] This provides

further evidence that the lens may register aging throughout the body, specifically processes which are affected by caloric restriction.

Epidemiologic evidence that the lens may reflect aspect of organismal aging comes from studies of age-related cataract. In large ophthalmologic cohorts in the United States, Bahamas, and Australia, cataract has been associated with cardiovascular disease, diabetes, obesity, inflammation, smoking, sunlight exposure (a DNA-damaging agent), and mortality.[135] Furthermore, there is a significant reduction in the age-specific rate and lifetime cumulative incidence of age-related cataract in individuals >90 years old with preserved cognition, a group exhibiting successful aging.[136] This evidence adds more weight to the potential usefulness of the lens as a biomarker of aging, though it is crucial to note that age-related cataract is a phenotype different from reduced lens transparency. Age-related cataract, or cataract surgery (a surrogate hard outcome), is a clinical phenotype derived from either physician diagnosis or medical billing codes whereas lens transparency is directly measured using optical devices. While age-related cataract is almost exclusively diagnosed in the latter half of life, lens transparency decreases beginning at age 14-15 years old.[137] Cataractous lenses are different from aged human lenses in gene expression patterns.[138] The clinical classification of the presence of “cataract” or “no cataract” also does not reflect the subtle morphological, histological, and biochemical changes that occur with lens aging, such as changes in lens curvature, thickness, or distensibility.[137,139-141] Subsequently, although previous studies of age-related cataract are supportive, new studies focused on lens transparency must be conducted to determine the validity of this different phenotype as a biomarker of aging.

From a feasibility standpoint, lens transparency is easily acquired because it is measured non-invasively and non-hazardously using optical devices such as Scheimpflug slit-lamp

photography.[137,142] It also provides a wide range, from 100% to 0% transparency, conceivably allowing fine risk stratification. Indeed, in the Aging Lens Substudy of the Cardiovascular Health Study, using Scheimpflug photography we detected a mean transparency of 76.61% (SD 17.75%) and a range of lens transparency from 8.29%-99.82% in approximately 60 Caucasian and African-American participants aged 79-88. This exemplifies the wide variability of transparency that exists even amongst a group of older individuals with a narrow age range, reinforcing the idea that transparency might depict the biologic variability seen in aging.

2.8.2: Measurement of lens transparency

Lens transparency can be assessed in several ways. Originally, grading systems were developed to indicate the severity of opacity in studies of cataract. These include the Age-Related Eye Disease Study (AREDS) criteria,[143] LOCS-III or the OCCCGS systems.[144,145] During a dilated eye exam, a trained grader compares the participant's lens to standardized photographs obtained with a photographic slit lamp. This adds to the complexity and duration of the eye exam and significantly increases cost, but it offers an accurate assessment of lens transparency. Depending on the system used and type of opacity examined (e.g., nuclear vs. cortical), measurements are ordinal, quasi-continuous, or fully continuous (e.g., 0-100% opacity).

Other imaging techniques, such as Scheimpflug photography, can provide a more accurate assessment of the nucleus with computer-aided determination of lens transparency using linear densitometry, can be acquired quickly, and can allow finer quantification of transparency, but they can be expensive and require additional training.[137,142,146] For Scheimpflug imaging, multiple measurements of each intact lens can be taken during a dilated eye exam. The optical density measurements are averaged to give a quantitative measurement of lens transparency for

each eye. Computerized assessment allows analysis of any part of the lens, which can provide more specific information, and should further minimize bias, though variability can result from some manual aspects of measurement such as marking which piece of the lens should be analyzed by the computer. Using Scheimpflug photography, Datiles et al. originally reported the intraclass correlation for the mean densities in the nucleus was 0.95, for the anterior cortex was 0.88, and for the posterior cortex was 0.84.[142] Excellent reproducibility has been reported by others.[137,146-150] Nuclear cataract lens density determined with Scheimpflug imaging has also been highly correlated with measurements taken using the LOCS-III grading criteria.[150]

2.8.3: Summary of previous research on the lens as a biomarker of aging

The biology of the lens makes it an attractive candidate marker of aging though it has been virtually unexplored in human aging studies. Studies in rodents, particularly those using crystallin gene knockout and caloric restriction, suggest the lens may register aspects of aging and that crystallins may play a key role in maintaining tissue integrity across tissues and throughout the lifespan, particularly in response to common chemical and molecular stressors. Human studies are dominated by those of diagnosed age-related cataract, a phenotype distinct from reduced lens transparency. In these studies, cataract has been associated with mortality, age-related chronic disease (particularly diabetes, cardiovascular disease, and potentially dementia), and behavioral risk factors for accelerated aging and disease (e.g., smoking). Studies of lens transparency measured with direct imaging have been concerned with developing the imaging techniques themselves and calculating their reproducibility and concordance with cataract grading systems rather than investigating the association of lens transparency to markers of aging and disease. These studies illustrate the high reliability of Scheimpflug photography and

that lens transparency decreases with age beginning in adolescence. To date, there is little epidemiologic data on the association of lens transparency and measurements of aging and disease. Because cataract, cataract surgery, and lens transparency are distinct phenotypes, it is unknown whether they will have similar associations with markers of aging and disease, i.e., whether these different phenotypes are exchangeable in studies of aging. Determining their relative validity requires calculating associations between cataract or lens transparency and markers of aging and disease in the same cohort using similar techniques. Generating this data may be the most worthwhile to vet lens transparency as a biomarker of aging.

2.9 Tables

Table 2.9.1: Examples of potential biomarkers of aging under investigation

Level of organization	Marker	Potentially measured aspect of aging	Potentially associated disease if low*	Potentially associated disease if high*
Genetic	DNA Acetylation	Transcriptional accessibility, epigenetic imprinting		
	DNA Methylation	Transcriptional accessibility, epigenetic imprinting		
	microRNA expression	Post-transcriptional regulation		
	Thymine dimer	DNA repair capacity, radiation exposure		Cancer, Xeroderma pigmentosum
	8-oxoguanine	DNA repair capacity, oxidation		Cancer
Molecular & Cellular	Interleukin-6	Inflammation, infection, oxidation		Cardiovascular disease, cancer, diabetes, frailty, sarcopenia, infection, cognitive decline
	C-reactive protein	Inflammation, infection, oxidation, liver function	Liver disease	Cardiovascular disease, cancer, diabetes, frailty, sarcopenia, infection, cognitive decline
	Telomere length	Inflammation, oxidation, cellular senescence, oncogenic potential	Idiopathic pulmonary fibrosis, bone marrow failure syndrome, dyskeratosis congenita, Werner's syndrome	Cancer
	p16	Cellular senescence, oncogenic potential	Cancer	
	Dehydroepiandrosterone sulfate	Sex steroid hormone reserve		
	Insulin-like growth factor	Energy metabolism, growth	Growth insufficiency	
	Advanced glycation end products	Non-enzymatic glycation and oxidation of proteins and lipids		Diabetes, cardiovascular disease, dementia
	Isoprostanes	Lipid peroxidation, oxidation		Neurodegeneration, coronary heart disease
	KLOTHO	Insulin sensitivity, Vitamin D regulation	Hyperphosphatemia, reduced bone	

Table 2.9.1 continued

			mineralization, kidney disease	
	Lipofuscin	Fatty acid oxidation, lysosomal digestion		Lipofuscinoses (neurodegeneration), liver disease, kidney disease
Tissue & Organ	Aortic calcification	Arteriosclerosis		Arteriosclerosis
	Pulse wave velocity	Arterial stiffness		Hypertension
	Forced expiratory volume	Pulmonary reserve, muscle strength	COPD	
	Brain volume	Cognitive reserve	Dementia	
	White matter hyperintensities	Integrity of white matter and subcortical tract connectivity		Hypertension, dementia
	Cystatin-C	Kidney function, inflammation		Kidney disease, cardiovascular disease
	Nerve conduction amplitude, monofilament sensitivity	Peripheral nerve function	Peripheral neuropathy, diabetes	
	Lens transparency	Maintenance of protein conformation, glycation, oxidation	Cataract	
	Lean mass	Sarcopenia	Frailty	
Organismal function	Gait speed	Muscle function, peripheral and central nervous system function, cardiopulmonary fitness		
	Digit Symbol Substitution Test	Processing speed, visual-motor function	Dementia	
	Fatigue	Energy metabolism, cardiopulmonary fitness, mental wellbeing		
	Activities of daily living	Mental and physical health		
	Frailty	Energy, strength, mental wellbeing		

*Associations may be correlative and/or causal. Most data is derived from human studies, though data for some markers (e.g., KLOTHO) is predominantly from animal models.

Table 2.9.2: Association of telomere length with age, sex, race, markers of health status, age-related chronic disease, and markers of inflammation and oxidation

Study	Sample	Study	Study design	Variable	Measurement method	Association	P-value
Batty 2009	1542 Caucasian men, age 45-64, without MI	WOSCOPS	Cross sectional	Age (year)	PCR	-8.5 bp	0.001
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Age (year)	Southern blot	-26 bp	<0.0001
Benetos 2001	193 Caucasian men and women, age 54-58, not on hypertensive medication		Cross sectional	Age (year)	Southern blot	r = -0.45 (Men), r = -0.48 (Women)	<0.0001, <0.0001
Benetos 2004	163 men, age 58-65, with chronic treated essential hypertension		Cross sectional	Age (year)	Southern blot	r = -0.25	<0.01
Brouilette 2003	203 cases with MI before age 50, 180 controls		Cross sectional	Age (year)	Southern blot	NA	<0.0001
Cherkas 2008	2401 Caucasian twins, age 18-81	TwinsUK	Cross sectional	Age (year)	Southern blot	r = -0.38	<0.001
Demissie 2006	327 Caucasian men, age 40-89	FHS	Cross sectional	Age (year)	Southern blot	r = -0.41	<0.0001
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Age (year)	Southern blot	-31 bp	<0.001
Hunt 2008	1742 Caucasian and 711 African-American, age 19-93	NHLBI-FHS, BHS	Cross sectional	Age (year)	Southern blot	-20 bp (Caucasian), -29 bp (African-American)	<0.0001, <0.0001
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Age (year)	PCR	r = -0.002 (Men), -0.230 (Women)	0.96, <0.001
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	Age (year)	PCR	-0.003 T/S (Men), -0.007 T/S (Women)	0.0002, <0.0001

Table 2.9.2 continued

Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Age (year)	PCR	r = -0.065 (Men), -0.055 (Women)	0.017, 0.038
Tang 2010	963 Chinese men and 904 women, age 65+		Cross sectional	Age (year)	PCR	Negatively correlated in men but not women	0.037, 0.78
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Male	Southern blot	-172 bp	<0.0001
Benetos 2001	193 Caucasian men and women, age 54-58, not on hypertensive medication		Cross sectional	Male	Southern blot	-280 bp (age-adjusted)	0.016
Hunt 2008	1742 Caucasian and 711 African-American, age 19-93	NHLBI-FHS, BHS	Cross sectional	Male	Southern blot	FHS: -120 bp (Caucasian), -260 bp (African-American). BHS: 10 bp (Caucasian), -130 bp (African-American)	FHS: <0.0001 BHS: 0.26
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Male	PCR	Shorter in men	<0.0001
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	Male	PCR	-0.041 T/S	0.0005
Tang 2010	963 Chinese men and 904 women, age 65+		Cross sectional	Male	PCR	-550 bp	<0.001
Chen 2009	472 Caucasian and 190 African American men and women, age 26-48	BHS	Cross sectional	Caucasian race	Southern blot	-541 bp (Men), -517 (Women)	<0.001, <0.001
Hunt 2008	1742 Caucasian and 711 African-American, age 19-93	NHLBI-FHS, BHS	Cross sectional	Caucasian race	Southern blot	FHS: -180 bp (Men), -320 bp	All <0.0001

Table 2.9.2 continued

						(Women). BHS: -500 bp (Men), -680 bp (Women)	
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	Caucasian race	PCR	-0.041 T/S (vs. African-American), -0.044 (vs. Hispanic)	0.025, 0.015
Batty 2009	1542 Caucasian men, age 45-64, without MI	WOSCOPS	Cross sectional	BMI (kg/m^2)	PCR	-1 bp	0.79
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	BMI (kg/m^2)	Southern blot	-4 bp (Men), -4 bp (Women)	0.41, 0.35
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	BMI (kg/m^2)	Southern blot	-10 bp	0.13
Hunt 2008	1742 Caucasian and 711 African-American, age 19-93	NHLBI-FHS, BHS	Cross sectional	BMI (kg/m^2)	Southern blot	r = -0.071	0.002
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	BMI (kg/m^2)	PCR	r = -0.041 (Men), -0.106 (Women)	0.35, 0.021
O'Donnell 2008	1062 Caucasian men and women, age 33-86	FHS	Cross sectional	BMI (kg/m^2)	Southern blot	r = -0.08	0.01
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	BMI (kg/m^2)	PCR	0.001 T/S	0.58
Valdes 2005	1122 Caucasian women twins, age 18-76	TwinsUK	Cross sectional	BMI (kg/m^2)	Southern blot	-0.077 TRF	0.031
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	BMI (kg/m^2)	PCR	Controls NS, Cases NS	0.61, 0.24
Batty 2009	1542 Caucasian men, age 45-64, without MI	WOSCOPS	Cross sectional	Ever smoking	PCR	35 bp	0.17
O'Donnell	1062 Caucasian men and	FHS	Cross	Current smoking	Southern blot	-130 bp	0.02

Table 2.9.2 continued

2008	women, age 33-86		sectional				
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Smoking (pack-year)	Southern blot	-20 bp (Men), 6 bp (Women)	0.16, 0.75
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Smoking (pack-year)	Southern blot	-2 bp	0.27
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Smoking (pack-year)	PCR	r = 0.084 (Men), -0.115 (Women)	0.46, 0.23
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	Smoking (pack-year)	PCR	-0.0007 T/S	0.054
Valdes 2005	1122 Caucasian women twins, age 18-76	TwinsUK	Cross sectional	Smoking (pack-year)	Southern blot	-0.11 TRF	0.045
Batty 2009	1542 Caucasian men, age 45-64, without MI	WOSCOPS	Cross sectional	Alcohol (units/week)	PCR	3 bp	0.93
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Alcohol (units/week)	Southern blot	-8 bp (Men), -7 bp (Women)	0.63, 0.68
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Physical activity (MET x times/wk)	Southern blot	25 bp (Men), -3 bp (Women)	0.15, 0.81
Cherkas 2008	2401 Caucasian twins, age 18-81	TwinsUK	Cross sectional	Physical activity (questionnaire)	Southern blot	-88 to -213 bp (depending on comparison)	<0.05
Roux 2009	981 Caucasian, African American and Hispanic men and women, age 45-84	MESA	Cross sectional	Leisure MET-mins (1000 s)	PCR	-0.009 T/S	0.13
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Weekly physical activity (kcal)	PCR	r = -0.036 (Men), -0.029 (Women)	0.19, 0.28
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Total cholesterol (10 mg/dL)	Southern blot	-2 bp (Men), 1 bp (Women)	0.75, 0.84

Table 2.9.2 continued

Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Total cholesterol (10 mg/dL)	PCR	r = 0.026 (Men), 0.070 (Women)	0.58, 0.13
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	Total cholesterol (10 mg/dL)	PCR	Controls NS, Cases NS	0.97, 0.18
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	HDL (10 mg/dL)	Southern blot	-20 bp (Men), 55 bp (Women)	0.15, 0.64
Chen 2009	472 Caucasian and 190 African American men and women, age 26-48	BHS	Cross sectional	HDL (10 mg/dL)	Southern blot	-31 bp (childhood), -44 bp (adulthood)	0.024, 0.058
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	HDL (10 mg/dL)	PCR	r = 0.111 (Men), 0.072 (Women)	0.053, 0.41
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	HDL (10 mg/dL)	PCR	Controls inverse association, Cases NS	0.046, 0.24
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	LDL (10 mg/dL)	Southern blot	-0.7 bp (Men), 3 bp (Women)	0.93, 0.70
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	LDL (10 mg/dL)	PCR	r = 0.040 (Men), 0.160 (Women)	0.49, 0.07
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Triglycerides (10 mg/dL)	Southern blot	2 bp (Men), 1 bp (Women)	0.21, 0.83
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Triglycerides (10 mg/dL)	PCR	r = -0.047 (Men), 0.004 (Women)	0.34, 0.95
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	Triglycerides (10 mg/dL)	PCR	Controls NS, Cases NS	0.48, 0.051
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Glucose (10 mg/dL)	Southern blot	4 bp (Men), -25 bp (Women)	0.85, 0.29

Table 2.9.2 continued

Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Glucose (10 mg/dL)	Southern blot	-15 bp	0.06
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Glucose (10 mg/dL)	PCR	r = -0.061 (Men), -0.045 (Women)	0.025, 0.089
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	Glucose (10 mg/dL)	PCR	Controls NS, Cases NS	0.72, 0.87
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	OGTT 0 min	PCR	r = -0.040 (Men), -0.001 (Women)	0.41, 0.98
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	OGTT 120 min	PCR	r = -0.202 (Men), -0.063 (Women)	0.045, 0.45
Demissie 2006	327 Caucasian men, age 40-89	FHS	Cross sectional	HOMA-IR score	Southern blot	r = -0.16	0.007
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Insulin (10 mg/dL)	Southern blot	-100 bp	0.009
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Insulin (10 mg/dL)	PCR	r = -0.026 (Men), 0.096 (Women)	0.65, 0.28
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Insulin (10 mg/dL)	PCR	r = -0.064 (Men), -0.069 (Women)	0.028, 0.014
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	SBP (10 mmHg)	Southern blot	15 bp (Men), -4 bp (Women)	0.34, 0.80
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	SBP (10 mmHg)	Southern blot	-34 bp	0.15
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	SBP (10 mmHg)	PCR	r = 0.064 (Men), -0.038 (Women)	0.15, 0.41
Yang 2009	Chinese, 379 controls and 388 hypertensive patients,		Cross sectional	SBP (10 mmHg)	PCR	Controls NS, Cases NS	0.09, 0.81

Table 2.9.2 continued

	age 30-80						
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	DBP (10 mmHg)	Southern blot	17 bp (Men), -5 bp (Women)	0.40, 0.82
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	DBP (10 mmHg)	Southern blot	-83 bp	0.06
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	DBP (10 mmHg)	PCR	r = 0.013 (Men), 0.010 (Women)	0.77, 0.82
Yang 2009	Chinese, 379 controls and 388 hypertensive patients, age 30-80		Cross sectional	DBP (10 mmHg)	PCR	Controls NS, Cases NS	0.08, 0.69
Benetos 2001	193 Caucasian men and women, age 54-58, not on hypertensive medication		Cross sectional	Pulse pressure (mmHg)	Southern blot	-3.7 / TRF fragment (Men), 0.4 / TRF fragment (Women)	0.005, NS
Nordfjall 2008	989 Caucasian, age 48-68	MDCC, NS-MONICA	Cross sectional	Pulse pressure (mmHg)	PCR	r = 0.075 (Men), -0.056 (Women)	0.09, 0.23
Benetos 2001	193 Caucasian men and women, age 54-58, not on hypertensive medication		Cross sectional	Pulse wave velocity (m/sec)	Southern blot	-0.5 / TRF fragment (Men), -0.2 / TRF fragment (Women)	0.047, NS
Benetos 2004	163 men, age 58-65, with chronic treated essential hypertension		Cross sectional	Presence of carotid artery plaque	Southern blot	-0.22 TRF length with presence of carotid plaque	<0.05
De Meyer 2009	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Presence of carotid artery plaque	Southern blot	-0.006 / TRF (Men), -0.202 / TRF (Women)	0.96, 0.09
De Meyer 2009	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Presence of femoral artery plaque	Southern blot	-0.101 / TRF (Men), -0.251 / TRF (Women)	0.28, 0.02
De Meyer	2509 Caucasian men and	Asklepios	Cross	Presence of	Southern blot	-0.083 / TRF	0.36,

Table 2.9.2 continued

2009	women, age 35-55, without overt CVD	study	sectional	carotid or femoral plaque		(Men), -0.243 / TRF (Women)	0.01
De Meyer 2009	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Carotid intima-media thickness (mm)	Southern blot	-0.003 / TRF (Men), -0.003 / TRF (Women)	0.54, 0.40
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Common carotid intima-media thickness (mm)	Southern blot	-260 bp	0.10
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Internal carotid intima-media thickness (mm)	Southern blot	-110 bp	0.07
O'Donnell 2008	1062 Caucasian men and women, age 33-86	CHS	Cross sectional	Internal carotid intima-media thickness (mm)	Southern blot	r = -0.055	0.15
De Meyer 2009	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	Femoral intima-media thickness (mm)	Southern blot	-0.004 / TRF (Men), 0.000 / TRF (Women)	0.73, 0.99
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	Ankle-brachial index	Southern blot	270 bp	0.09
Mainous 2010	325, aged 40–64, free of diagnosed diabetes, CHD, stroke and cancer		Cross sectional	Coronary artery calcium	PCR	r = -0.15	0.009
Brouilette 2003	203 cases with MI before age 50, 180 controls		Cross sectional	MI before age 50	Southern blot	-300 bp	<0.0001
Honig 2006	125 Demented cases, 132 Non-demented controls, age 66-103	WHICAP	Cross sectional	Dementia	PCR	-0.058 T/S	0.034
Honig 2006	125 Demented cases, 132 Non-demented controls, age 66-103	WHICAP	Cross sectional	ApoE4 allele (≥1)	PCR	-0.042 T/S	NS
Yaffe 2009	2734 Non-demented Caucasian and African-American men and women,	HABC	Cross sectional, longitudinal	ApoE4 allele (≥1)	PCR	Not available	0.16

Table 2.9.2 continued

	age 70-79						
Yaffe 2009	2734 Non-demented Caucasian and African-American men and women, age 70-79	HABC	Cross sectional, longitudinal	3MS	PCR	Not available	0.28
Yaffe 2009	2734 Non-demented Caucasian and African-American men and women, age 70-79	HABC	Cross sectional, longitudinal	DSST	PCR	Not available	0.02
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Hip BMD	PCR	r = -0.017 (Men), -0.007 (Women)	0.53, 0.79
Tang 2010	963 Chinese men and 904 women, age 65+		Cross sectional	Hip BMD	PCR	2 bp (Men), 0 bp (Women)	0.42, 0.99
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	Femoral neck BMD	PCR	r = -0.003 (Men), -0.016 (Women)	0.92, 0.55
Tang 2010	963 Chinese men and 904 women, age 65+	TwinsUK	Cross sectional	Femoral neck BMD	PCR	3 bp (Men), -1 bp (Women)	0.11, 0.46
Valdes 2007	2150 Caucasian women twins, age 18-80	TwinsUK	Cross sectional	Femoral neck BMD	Southern blot	r = 0.040	0.052
Valdes 2007	2150 Caucasian women twins, age 18-80	TwinsUK	Cross sectional	Forearm BMD	Southern blot	r = 0.054	0.013
Valdes 2007	2150 Caucasian women twins, age 18-80	TwinsUK	Cross sectional	Spine BMD	Southern blot	r = 0.058	0.005
Bekaert 2007	2509 Caucasian men and women, age 35-55, without overt CVD	Asklepios study	Cross sectional	IL-6 (highest half)	Southern blot	-133 bp (Men), -83 bp (Women)	<0.01, >0.05
Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	IL-6 (pg/mL)	Southern blot	-50 bp	0.03
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	IL-6	PCR	r = -0.074 (Men), -0.008 (Women)	0.008, 0.77

Table 2.9.2 continued

Fitzpatrick 2007	419 men and women, age 65-93	CHS	Cross sectional	CRP (mg/L)	Southern blot	-59 bp	0.02
Sanders 2009	2750 Caucasian and African-American men and women, age 70-79	HABC	Cross sectional	CRP	PCR	r = -0.051 (Men), 0.004 (Women)	0.063, 0.88
Demissie 2006	327 Caucasian men, age 40-89	FHS	Cross sectional	Isoprostane urinary 8-epi-$PGF_{2\alpha}$	Southern blot	r = -0.16	0.005
Richards 2008	1319 Caucasian twins, age 18-81	TwinsUK	Cross sectional	Homocysteine (highest tertile)	Southern blot	-111 bp	0.004

Study acronyms: BHS, Bogalusa Heart Study. CHS, Cardiovascular Health Study. FHS, Framingham Heart Study. HABC, Health, Aging, and Body Composition Study. MDCC, Malmö Diet and Cancer Cohort. MESA, Multi-Ethnic Study of Atherosclerosis. NS-MONICA, Northern Sweden Monitoring of Trends and Determinants in Cardiovascular Diseases. NHLBI-FHS, National Heart, Lung, and Blood Institute Family Heart Study. WHICAP, Washington Heights-Inwood Columbia Aging Project. WOSCOPS, West of Scotland Coronary Prevention Study.

3.0 SPECIFIC AIMS

LTL and lens transparency require further validation as biomarkers of aging and/or age-related chronic disease. We aim to overcome limitations of previous studies to advance understanding of the usefulness of these markers.

Leukocyte telomere length

Most studies of LTL concern only clinically diagnosed disease, but disease builds over time and can exist in subclinical, preclinical, undiagnosed, or misdiagnosed states. Depending on the selection criteria to identify a study cohort (e.g., demographics, function, health status), the burden of subclinical disease varies widely, but in general, in cohorts of older individuals without clinically diagnosed disease, the prevalence and severity range of subclinical disease can be substantial when assessed using noninvasive methods.[151-154] Importantly, undiagnosed disease powerfully predicts incident adverse events independent of diagnosed disease.[13,151-157] Subsequently, previous studies relying on yes/no disease categorization may have missed associations between LTL and age-related disease. Moreover, studies of LTL and age-related disease have focused on a single organ or biological system rather than "disease burden" across systems. This distinction is relevant since LTL apparently records systemic burden of inflammation and oxidative stress and may report a synchrony of aging throughout the body. Fortunately, in the Cardiovascular Health Study, an index of disease burden has been constructed

and validated as a predictor of mortality and incident disability independent of age and diagnosed disease.[13]

Specific aim 1: *Using a more encompassing depiction of health, disease burden, which can be defined as the sum of noninvasively measured markers of structure or function in different organ systems, we determined if: 1) shorter LTL is associated with greater age-related disease burden; 2) shorter LTL is less strongly associated with disease in individual systems, or diagnosed chronic conditions (cardiovascular disease, stroke, pulmonary disease, diabetes, kidney disease, arthritis, or depression).*

Lens transparency

It is unknown whether lens transparency measured on a continuous scale, diagnosis of cataract, or cataract surgery are equivalently useful to investigate the lens as a potential biomarker of aging. Determining which measure of lens transparency is best will enable researchers to more effectively validate lens transparency as a biomarker. To explore these different phenotypes, we studied the association of the lens with LTL in two groups of individuals: 1) a large cohort of community-dwelling older adults, the Health, Aging and Body Composition Study (Health ABC), in which participants self-reported physician diagnosis of cataract and cataract surgery; 2) an ophthalmic sub-study of Health ABC, the Age-Related Maculopathy Ancillary Study (ARMA), which had more sensitive measurement of cataract severity using AREDS grading criteria whereby a trained examiner graded cataract severity using standard photos during a slit lamp exam. Using these two groups we were able to distinguish lens transparency, illustrated by very clear lenses according to AREDS grading criteria, from cataract, illustrated by self-report of physician diagnosis of cataract or cataract surgery.

Specific aim 2: *To determine if 1) longer LTL is associated with a lower prevalence of self-reported cataract or cataract surgery; 2) longer LTL is associated with a lower incidence of cataract surgery; and 3) longer LTL is associated with a lower odds of lens opacity measured using AREDS grading criteria.*

Although the loss of lens transparency correlates with increasing age, it had not been examined with respect to the presence of other markers of aging or subclinical diseases. For better enumeration of the association between directly-measured lens transparency and aging or disease, we created the Aging Lens Sub-study to the Healthy Women Study. The Healthy Women Study was a community-based longitudinal cohort study that began in 1983-84 and was refunded for a fourth examination of the determinants of risk factor changes among women during peri- and postmenopause. When participants returned for continued evaluation, we added a measure of lens transparency using Scheimpflug photography, which uses linear densitometry to provide a quantitative measure of transparency. Again, to distinguish lens transparency from cataract, we also recorded self-report of cataract surgery. We conducted a cross-sectional study of the association of current measures of aging and disease with lens transparency and cataract surgery, and to more carefully investigate temporality, we conducted a retrospective longitudinal study by testing the association of previous measures of aging and disease with current lens transparency or history of cataract surgery.

Specific aim 3: *To determine the association of lens transparency and cataract surgery to 1) risk factors for coronary atherosclerosis, the extent of noninvasively measured coronary atherosclerosis, fasting plasma glucose or insulin; 2) markers of cognitive function.*

4.0 PAPER 1: LEUKOCYTE TELOMERE LENGTH IS ASSOCIATED WITH NONINVASIVELY MEASURED AGE-RELATED DISEASE: THE CARDIOVASCULAR HEALTH STUDY

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4.1 ABSTRACT

Background: Most studies of leukocyte telomere length (LTL) focus on diagnosed disease in one system. A more encompassing depiction of health is disease burden, defined here as the sum of noninvasively measured markers of structure or function in different organ systems. We determined if: 1) shorter LTL is associated with greater age-related disease burden; 2) shorter LTL is less strongly associated with disease in individual systems, or diagnosed chronic conditions (cardiovascular disease, stroke, pulmonary disease, diabetes, kidney disease, arthritis, or depression**)**.

Methods: LTL was measured by Southern blots of terminal restriction fragment length. Age-related disease was measured noninvasively and included carotid intima-media thickness, lung vital capacity, white matter grade, cystatin-C, and fasting glucose; each graded 0 (best tertile), 1 (middle tertile), or 2 (worst tertile); and summed (0 to 10) to estimate disease burden. Of 419 participants randomly selected for LTL measurement, 236 had disease burden assessed (mean (SD) age 74.2 (4.9) years, 42.4% male, 86.8% white, 13.2% black).

Results: Mean (SD) LTL was 6312 (615) bp and disease score was 4.7 (2.1) points. A SD higher disease score (β(SE) = -132(47) bp, P<0.01), age (β(SE) = -107(46), P=0.02), or carotid thickness (β(SE) = -95(40) bp, P=0.02) was associated with shorter LTL but diagnosed

conditions or number of conditions were not associated with LTL. Disease score attenuated the effect of age on LTL by 35%.

Conclusions: LTL was associated with a characterization of age-related disease burden across multiple physiologic systems which was comparable to, but independent of, its association with age.

4.2 BACKGROUND

Although biologic age may be impossible to define completely, it will be better understood by uncovering biomarkers of aging. In the future these biomarkers might guide preventive or therapeutic interventions before clinical onset of age-related disease. Leukocyte telomere length (LTL) might be such a biomarker because it ostensibly records the accruing burden of inflammation and oxidative stress, processes thought to contribute to aging and disease pathogenesis.[23,25,158] LTL undergoes progressive shortening with age and in the general population it is comparatively short in individuals with atherosclerosis or those at risk for this aging-related disease.[159] The evidence for LTL as an overall biomarker of aging remains unclear, though, due to variation in the strength of detected associations, differences in results based on measurement method and selected outcome, lack of data coupling longitudinal measurements of LTL and aging phenotypes, and theoretical considerations of whether a single marker can accurately and strongly record aging across the lifespan.[26,101,160]

Most studies of LTL concern only clinically diagnosed disease, but in cohort studies of older individuals without clinically diagnosed disease, the prevalence and severity range of subclinical disease can be substantial when assessed using several noninvasive methods.[151-154] Furthermore, undiagnosed disease can powerfully predict incident adverse events independent of diagnosed disease.[151-157] Defining disease in categorical (yes/no) terms is imprecise, whereas using quantitative biomarkers might provide a more realistic picture of age-related disease load.

Therefore previous studies relying on disease categorization may have missed associations between LTL and age-related disease.

Most studies exploring the links between LTL and age-related disease have focused on a single organ or biological system rather than "disease burden" across systems. Here, we define "disease burden" as the sum of noninvasively measured markers of age-related dysfunctions in structure or physiology of different organ systems. This distinction is relevant since LTL apparently records systemic burden of inflammation and oxidative stress. Thus, a study of LTL and disease burden is warranted to clarify the validity of LTL as a biomarker of aging or age-related disease.

Recently, Newman et al. developed a physiologic index of comorbidity (index), a 10-point scale that tabulates the severity of age-related chronic disease using noninvasive tests of the vasculature, lungs, kidneys, brain, and glucose metabolism.[13] This estimates an individual's disease burden regardless of whether disease is clinically recognized. Given its more continuous range it demonstrated the spectrum of chronic disease in a general population of community-dwelling older adults. The index explained 40% of the age effect on mortality risk and illustrated that high disease burden, even when clinically unrecognized, was significantly associated with mobility limitation and failure to complete activities of daily living. The index appears to be a valid measure of disease burden and stratifies individuals into a wide range of risk.

Using data on LTL and age-related disease burden from the Cardiovascular Health Study (CHS), we conducted this analysis to test two hypotheses: 1) shorter LTL is associated with greater age-related disease burden; 2) shorter LTL is less strongly associated with disease in individual systems, or diagnosed chronic conditions (cardiovascular disease, stroke, pulmonary disease, diabetes, kidney disease, arthritis, or depression).

4.3 RESEARCH DESIGN AND METHODS

4.3.1: Population

The CHS is an ongoing community-based study of cardiovascular risk in 5888 men and women over the age of 65 years, from four regions of the United States.[161] The cohort was enrolled in 1989-1990 (N=5201) and was supplemented with added minority recruitment in 1992-1993 (N=687). Participants and eligible household members were identified from a random sample of Medicare enrollees at each field center. Participants were ≥65 years old and to be eligible they could not have cancer under active treatment, could not be wheelchair- or bed-bound in the home, and did not plan to move out of the area within 3 years. Using blood samples from the 1992-1993 examination, 419 CHS participants were originally randomly selected for LTL measurement. Of these, 236 had index scores available and form the study population for this analysis (mean (SD) age 74.2 (4.9) years, range 65-91 years, interquartile range 71-77 years, 42.4% male, 86.8% white, 13.2% black). Participants without index scores available were less educated (61.2% with less than a college education vs. 49.6% with less than a college education, P=0.02), less likely to be white (76.0% vs. 86.4% vs, P<0.01), and had high fibrinogen (334 mg/dL vs. 316 mg/dL, P=0.01). They had similar LTL (6312 bp vs. 6367 bp, P=0.36), age (74.2 years vs. 74.2 years, P=0.97), gender (39.3% men vs. 42.4% men, P=0.53), smoking history (53.6% ever smoked vs. 51.3% ever smoked, P=0.64), body mass index (BMI) (27.8 kg/m^2 vs. 26.9 kg/m^2, P=0.06), C-reactive protein (CRP) (3.02 mg/L vs. 2.59 mg/L, P=0.12), and total cholesterol, high density lipoprotein cholesterol, and triglycerides (all P>0.3), indicating that

selection bias was likely minimal. The CHS is approved by the Institutional Review Boards of all participating institutions.

4.3.2: Physiologic index of comorbidity

The instruments and methods used to construct the index have been described previously.[13] Briefly, the clinical examination conducted in 1992-1993 included cardiovascular and pulmonary function tests, blood tests for glucose tolerance and kidney function, and a brain MRI. The choice of tests to include in the index was based on previous reports that each is individually an important predictor of mortality, and that each represents a major, common age-related chronic disease.[161,162] Carotid ultrasound was obtained in the left and right internal and common carotid arteries to assess near and far wall thicknesses and Doppler flow. The mean of the maximum wall thickness of the internal carotid artery was used to represent the extent of vascular disease.[163] Spirometry was conducted according to the standards of the American Thoracic Society.[161] Fasting glucose was assessed as described previously.[164] Cystatin-C, a serum marker of glomerular filtration rate, was assessed using a BNII nephelometer that used a particle-enhanced immunonephelometric assay.[165] Brain MRI was obtained according to a standard scanning protocol and data was interpreted at a central MRI Reading Center by a neurologist trained in a standardized protocol.[166] The white matter grade score was used to indicate small-vessel vascular disease in the brain.[162]

To construct the index, each of the five measures was divided into three groups with the best values classified as 0 and the worst as 2.[13] Although the choice of cut points was arbitrary, the best score of ‘‘0’’ was generally found to represent a healthy normal value, and values of ‘‘2’’ were in the range of individuals with diagnosed chronic disease. Individual scores were

summed for a total score ranging from 0 to 10. For the carotid wall thickness, tertile cut points were scored as 0: 0.60–1.06 mm, 1: 1.06–1.53 mm, 2: 1.53– 3.94 mm. Because there was little overlap between men and women for forced vital capacity, tertile cut points for forced vital capacity were sex-specific (Women: 0: 2.6–3.8 L, 1: 2.2–2.6 L, 2: 0.6–2.2 L; Men: 0: 3.9–6.5 L, 1: 3.2–3.9 L, 2: 0.3–3.2 L). Tertile cut points for cystatin-C were scored as 0: 0.6–1.0 mg/L, 1: 1.0–1.1 mg/L, 2: 1.1–3.5 mg/L. For white matter grade, tertile cut points were scored as 0: 0–1 units, 1: 2 units, 2: 3–9 units on the 0–9 ordinal scale. Fasting glucose was the only measure not classified by tertile. Although results were similar, for clinical interpretation, this presentation uses cut points classified according to clinical cut points defined by the American Diabetes Association (0: <100 mg/dL, 1: 100–126 mg/dL, 2: >126 mg/dL).[167]

4.3.3: Terminal restriction fragment length

CHS participants eligible for LTL measurement were randomly selected from those who completed the 1992-1993 clinic examination, consented to DNA preparation/use, had at least 12 ug of DNA available, and had stored leukocytes for additional DNA preparation. The integrity of the DNA was assessed through electrophoresis on 1.0% agarose gels and LTL was measured as the mean length of the terminal restriction fragments (TRFs) by the Southern blot method previously described.[57,103] Each sample was analyzed twice for LTL measurement (on different gels on different occasions), and the mean was used for statistical analyses. The Pearson correlation coefficient for the duplicates of this sample was 0.97, with an average inter-assay coefficient of variation (CV) of 1.5%. The laboratory conducting the TRF length measurements was blinded to all characteristics of participants.

4.3.4: Demographic, behavioral health, and clinical disease variables

Covariates were selected for their documented or proposed association with age-related diseases included in the index or LTL. Age, sex, race (black, white, or other), and education were ascertained by self-report. Smoking was assessed by a standardized interview.[168] Blood pressure, height, and weight were assessed by standardized protocols. BMI was calculated as kilograms per meter squared. CRP was assessed with a high-sensitivity enzyme-linked immunosorbent assay.[169] The inter-assay CV was 5.50%. Plasma fibrinogen was measured using a semiautomated modified clot-rate method. The mean monthly CV for the fibrinogen assay was 3.09%. For consistency, we tabulated clinically diagnosed chronic conditions using the same methods as in the original report of the index.[13] Pulmonary disease, diabetes, kidney disease, and arthritis were assessed by self-report of physician diagnosis to depict what would be diagnosed disease. Depression was defined on the basis of a score >10 on a modified 10-item CES-D score.[170,171] Reports of cardiovascular disease and stroke were confirmed by review of medications and medical records.[161] Using this information, a count of diagnosed chronic conditions was constructed for each person with a maximum of 7 for these conditions: cardiovascular disease, stroke, pulmonary disease, diabetes, kidney disease, arthritis, and depression.[13]

4.3.5: Statistical analysis

To depict population characteristics and identify potential confounders, we evaluated the association between covariates and the index or LTL using a test for trend, χ^2-test or Fisher's exact test, where appropriate. A lowess smoothed curve was used to inform modeling of the association of the index to LTL. It implied a linear form, which was supported by a goodness-of-

fit test after model building. We hypothesize that LTL is a predictor of disease outcomes, but set up this cross-sectional analysis with LTL as the dependent variable in order to express association in terms of base pairs of LTL. This is consistent with most other reports in the literature. Furthermore, it allowed us to compare age and disease directly in their associations with LTL. Subsequently, we built a series of general linear models using the index as the predictor and LTL as the outcome adjusting for covariates as follows: unadjusted model; model 1 (adjusted for age, gender, and race); model 2 (model 1 plus education, BMI, and current smoking status); model 3 (model 2 plus fibrinogen and natural logarithm of CRP); model 4 (model 3 plus the number of diagnosed chronic conditions). In the full model, we tested for interactions between the index and each covariate. Variance inflations factors were calculated for model covariates and confirmed that collinearity was minimal (all VIFs <2). The percent variance in LTL independently explained by the index or age, after adjustment for covariates, was determined using the part correlation.[172]

To test if LTL was associated with disease in individual physiologic systems we calculated the Pearson correlation coefficient between LTL and each index component. We also built general linear models with each component predicting LTL adjusting for age, gender, and race. To see if mean LTL was different by presence or absence of diagnosed chronic conditions we used the two-sample T-test or Wilcoxon rank sum test. In model 4 (above), we also replaced the count of diagnosed chronic conditions with the conditions themselves to test if they were associated with LTL independent of potential confounders. For all analyses we used a two-sided alpha of 0.05 to determine significance and SAS 9.2 (SAS Institute, Cary, NC).

4.4 RESULTS

Mean (SD) LTL was 6312 (615) bp and index score was 4.7 (2.1) points. Higher disease burden was associated with being older, higher CRP and fibrinogen, smoking, coronary heart disease, and diabetes (Table 4.7.1). LTL was associated with age (Pearson r = -0.269, P<0.001) and smoking (current smoker 6260 bp vs. past smoker 6196 bp vs. never smoker 6421 bp, P=0.03). Though LTL was not associated with gender (women 6374 bp vs. men 6226 bp, P=0.07) and race (whites 6299 bp vs. blacks 6392 bp, P=0.43), trends were as expected. LTL was not associated with BMI, education, CRP, fibrinogen, or a count of diagnosed chronic conditions (all P>0.1).

The index was significantly inversely correlated with LTL (Pearson r = -0.296, P<0.001). In an unadjusted model, each SD higher index score was associated with a 183 bp shorter LTL (P<0.001) (Table 4.7.2). For comparison, when modeled by itself a SD higher age was associated with a 165 bp shorter LTL (P<0.001). In a model with only the index and age, the two factors attenuated the association of each other to LTL (index β(SE) = -134(42) bp per SD, P<0.01; age β(SE) = -102(44) bp per SD, P=0.02), but each remained significantly associated with LTL.

In the fully adjusted model, a SD higher index score was associated with a 132 bp shorter LTL (P<0.01). A SD higher age was associated with a 107 bp shorter LTL (P=0.02), illustrating the association of disease burden was independent of, and similar to, the association of age with LTL. The association of a SD in the index score (2.1 points) was equivalent to the association of 5.9 years of age with LTL. When the index was removed from the full model, the effect of age

increased from -107 bp per SD to -165 bp per SD (P<0.001), indicating the index attenuated the effect of age by 35%. There was no interaction with the index and other covariates were not significantly associated with LTL. Disease burden independently accounted for 3.1% of the variance in LTL, while age independently accounted for 2.1% of the variance in LTL.

Regarding continuous components of the index, higher carotid artery thickness, white matter grade, and serum cystatin-C, but not forced vital capacity or serum fasting glucose, were significantly correlated with shorter LTL (Table 4.7.3). In linear models adjusted for age, gender, and race, only carotid thickness was independently associated with LTL, though white matter grade was borderline associated with LTL (Table 4.7.3). After further adjustment for BMI, education, smoking, CRP, fibrinogen, and a count of diagnosed chronic conditions, the effect of a SD higher carotid thickness (β(SE) = -95(40) bp, P=0.02) was about 3/4 that of the effect of the index. Including all index components in the same model resulted in similar findings, illustrating that the components were operating relatively independently.

Regarding diagnosed chronic conditions, LTL was shorter with coronary heart disease (6152 bp vs. 6351 bp, P=0.04) but not with other conditions (Figure 4.8.1). When age was adjusted for, coronary heart disease was no longer associated with LTL (6163 bp vs. 6348 bp, P=0.06), and the association was attenuated more with additional adjustment for race, smoking, BMI, education, and CRP (6196 bp vs. 6343 bp, P=0.14). For comparison, when the index was dichotomized, those in the unhealthier half of the index (5-10 points) had markedly shorter LTL than those in the healthier half of the index (0-4 points) (6102 bp vs. 6525 bp, P<0.001) (Figure 4.8.1).

Because carotid thickness seemingly accounted for a substantial portion of the association of the index to LTL we conducted a post-hoc analysis removing carotid thickness

from the index to determine if non-vascular disease burden remained independently associated with LTL. In an unadjusted model, a SD in the index without carotid thickness was associated with LTL (β(SE) = -147(39) bp, P<0.001). Adjustment for age, gender, and race attenuated the association (β(SE) = -95(43) bp, P=0.03). Additional adjustment attenuated the association to borderline non-significance (β(SE) = -89(47) bp, P=0.06).

4.5 DISCUSSION

We hypothesized that shorter LTL is associated with greater age-related disease burden, and that shorter LTL is less strongly associated with disease in individual systems, or diagnosed chronic conditions. Our cross-sectional analysis shows that LTL was indeed strongly and independently associated with disease burden, a characterization of age-related chronic disease in five major physiologic systems independent of clinical diagnosis. In contrast, LTL was less strongly associated with disease in individual systems, except possibly carotid thickness, and was not associated with diagnosed chronic conditions or a count of diagnosed chronic conditions. Based on this data, we advocate that in older adults LTL may be associated most with processes which contribute to atherosclerosis and/or hypertension, and perhaps additional processes which contribute to structural and physiological changes in other organs, such as accumulation of white matter hyperintensities. It is possible that LTL might indicate widespread incremental changes in structure or function in older adults, but most powerfully vascular changes.

These results provide insight on measuring the biologic age of a human. LTL is a proposed indicator of aging so it is standard to correlate LTL with age. We found that a SD in the physiologic index or chronologic age had a similar, independent association with LTL. Age attenuated the effect of the index substantially but the index in turn accounted for 35% of the age effect on LTL. Although one could claim that the association of the index to LTL is partially explained by age, another perspective is that the association of age to LTL is partially explained by the index. These findings lend credence to the use of noninvasive tests of physiologic

structure and function to monitor the integrity of an aging adult. It demonstrates that novel biomarkers, such as LTL, hold great promise for unraveling the underlying aging process.

The significant association between LTL and carotid thickness and weaker associations with other systems may illustrate how organs age differently. Carotid thickness reflects seemingly normal medial thickening with age, vascular remodeling in response to hypertension, and atherosclerosis. The literature describes a robust association between LTL and atherosclerosis.[33,51,53,54,56,57,63,71-75] In our analysis carotid thickness did not account for the complete association between LTL and disease burden. White matter grade may illustrate normal brain aging in addition to hypertension and dementia. Using techniques other than MRI, some reports suggest that LTL may register cognitive aging or dementia. Notably, in 382 women not diagnosed with dementia or cognitive impairment, shorter LTL was independently associated with worse memory and learning.[81] This is supported by data from the Nurses' Health Study[82] and a hospital-based case-control study,[83] but not by a cohort of 449 inpatients in which there was no difference in LTL between patients who were cognitively normal, had mild cognitive impairment, or were demented.[84] A report from the Health Aging and Body Composition study, where LTL was examined in association with cognitive function in 2734 non-demented community dwelling older adults, provides inconclusive results.[85] The data presented here may support the idea of organ-specific aging or disease progression rather than simultaneous decline in organ function. It should be noted, though, that different magnitudes of associations between LTL and select tissues may depend on the specificity of markers for those tissues, and also differences in power to detect significant associations using dichotomized or classified outcomes vs. continuous outcomes with a wider variance. Future studies must carefully compare results in

light of measurement methods which focus on different points along the aging-disease continuum.

Our data must also be interpreted in light of our construct for disease burden. We wanted to count major physiologic systems only once and not give extra weight to cardiovascular disease. These conditions were selected because they are the most common chronic conditions in older adults. In our previous work we showed quite clearly that these five systems characterize mortality risk quite comprehensively.[13] Furthermore, we showed that a count of diagnosed conditions, including those not ascertained by the five-component physiologic index, can identify high risk individuals but not the lowest risk individuals, as is achieved by the five-point physiologic index. The lack of association of LTL with individual diagnosed conditions is likely due to the lower power of using dichotomous variables, but also due to the fact that there is a wide range of measurable disease in those not yet diagnosed. The partial overlap could potentially place the physiologic index at a disadvantage in prediction compared to a comorbidity count, which is addressed more fully in the prior publication.[13] Other approaches for measuring disease burden can be argued and more work is needed to determine the optimal balance between information gained and maintaining a biologically logical construct.

This study is strengthened by the use of Southern blot, the gold standard, to measure LTL. Although LTL was measured in a random sample of the CHS cohort, which should minimize selection bias, we acknowledge the potential for selection bias which may have resulted from using a subset of this random sample. Nonetheless, the original report from the CHS random LTL sample also found no association with BMI, fasting glucose, and hypertension, and similar trends with carotid thickness, gender, CRP, and smoking.[57] We found LTL was longer in women and blacks, and non-significance was likely due to our relatively

small sample size. Though there is a potential for survival bias when studying older individuals, this survival bias would most likely have the effect of truncating the distribution of telomere length at the short end and disease at the high end. If the association were weaker or stronger at the extremes or if there were a threshold, we would have less power to detect it. Therefore the reported associations here are likely to be conservative. Studies across a wider age range could be more powerful. Carotid thickness is a continuous variable and depicts a wider range of disease than dichotomous classification of yes/no presence of disease – subsequently, it is also possible that we identified a significant association of LTL to carotid thickness but not diagnosed coronary disease or cerebrovascular disease due to differences in power. The rich data available in the CHS allowed us to adjust for confounders identified in the literature, but we acknowledge residual confounding is possible. Finally, because the CHS is a general cohort study of community-dwelling older adults, the results may be relatively generalizable, though they should be replicated in other larger samples, particularly to achieve a higher prevalence of some rarer diagnosed chronic conditions like cerebrovascular disease and kidney disease, and in younger individuals to see if this association persists in midlife, which may increase confidence in LTL's ability to record aging or pathogenesis of age-related disease across the lifespan.

In conclusion, we found that LTL was strongly associated with disease burden and less strongly or not at all with disease in individual systems or diagnosed chronic conditions. Research that employs more encompassing measures of age-related disease, such as the physiologic index, may be most useful for identifying markers of biologic age.

4.6 ACKNOWLEDGEMENTS

Authors' contributions: conception and design (JLS, ABN); acquisition of data (AF, AMA, AA, MK, ABN); analysis and interpretation of data (JLS, AF, RMB, ABN); drafting of the manuscript (JLS, ABN); critical revision of the manuscript (JLS, AF, RMB, AMA, AA, LFF, TBH, ABN); statistical analysis (JLS, RMB); obtaining funding (AF, AA, ABN); administrative, technical, or materials support (ABN); supervision (ABN).

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4.7 TABLES

Table 4.7.1: Characteristics of study participants by physiologic index score group: the Cardiovascular Health Study, 1992-1993 examination

	Physiologic Index Score Group				
Characteristics	**0-2 (N=40)**	**3-4 (N=77)**	**5-6 (N=67)**	**7-10 (N=52)**	**P***
Demographics					
Age, y, mean (SD)	71.1 (2.8)	72.9 (3.6)	75.6 (5.6)	76.8 (4.8)	<0.001
Male gender, n (%)	10 (25.0)	35 (45.5)	32 (47.8)	23 (44.2)	0.11
Black race, n (%)	8 (20.0)	8 (10.4)	7 (10.5)	9 (17.3)	0.35
Behavioral risk factors					
Education less than college, N (%)	18 (45.0)	32 (41.6)	39 (58.2)	28 (53.9)	0.20
BMI, kg/m^2, mean (SD)	26.8 (3.4)	26.6 (4.0)	27.0 (4.5)	27.4 (3.8)	0.19
Smoking status					
Current, *n* (%)	4 (10.0)	3 (3.9)	10 (14.9)	8 (15.4)	0.03
Past, *n* (%)	15 (37.5)	26 (33.8)	33 (49.3)	23 (44.2)	
Never, *n* (%)	21 (52.5)	48 (62.3)	24 (35.8)	21 (40.4)	
Diagnosed chronic health conditions					
Coronary heart disease, *n* (%)	3 (7.5)	12 (15.6)	18 (26.9)	14 (26.9)	0.04
Cerebrovascular disease, *n* (%)	1 (2.5)	2 (2.6)	4 (6.0)	3 (5.8)	0.69
Diabetes, *n* (%)	1 (2.5)	4 (5.2)	16 (23.9)	14 (26.9)	<0.001
Obstructive lung disease, *n* (%)	12 (30.0)	20 (26.0)	24 (35.8)	14 (26.9)	0.59
Kidney disease, *n* (%)	0 (0)	1 (1.3)	2 (3.0)	1 (1.9)	0.83
Arthritis, *n* (%)	18 (45.0)	41 (53.3)	30 (44.8)	19 (36.5)	0.31
Depression (CES-D >10), *n* (%)	2 (5.0)	8 (10.4)	7 (10.5)	7 (13.5)	0.62
No. of conditions, 0-7, mean (SD)	0.9 (0.8)	1.1 (0.9)	1.5 (1.0)	1.4 (1.1)	<0.01
Inflammation/coagulation markers					
CRP, mg/l, mean (SD)	2.73 (2.75)	4.19 (8.64)	4.17 (4.07)	6.25 (9.81)	<0.001
ln(CRP), mean (SD)	0.671 (0.788)	0.835 (0.945)	1.03 (0.922)	1.23 (1.02)	<0.001
Fibrinogen, mg/dl, mean (SD)	300 (54.8)	309 (61.8)	319 (55.6)	335 (59.8)	<0.001

BMI, body mass index. CES-D, Center for Epidemiologic Studies Depression scale. CRP, C-reactive protein.

*P-value from test of trend, chi-square test, or Fisher's exact test.

Table 4.7.2: Linear regression models of the association of leukocyte telomere length to physiologic index score

	Age itself*		Index itself*		Model 1[†]		Model 2[‡]		Model 3[§]		Model 4[‖]	
Covariate	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**P**	**β (SE)**	**P**
Index score, 1 SD			-183 (38)	<0.001	-132 (42)	<0.001	-130 (45)	<0.01	-124 (46)	<0.01	-132 (47)	<0.01
Demographics												
Age, 1 SD	-165 (39)	<0.001			-102 (44)	0.02	-106 (45)	0.02	-111 (46)	0.02	-107 (46)	0.02
Male gender					-123 (77)	0.11	-118 (78)	0.13	-128 (80)	0.11	-122 (80)	0.13
Black race					73 (111)	0.51	92 (115)	0.42	88 (115)	0.45	84 (115)	0.47
Behavioral risk factors												
Education less than college							0 (78)	0.99	-4 (79)	0.96	-12 (81)	0.88
Body mass index, 1 SD							-9 (40)	0.82	-9 (42)	0.83	-15 (43)	0.73
Current smoking status							-36 (129)	0.78	-46 (131)	0.72	-45 (131)	0.73
Inflammation and coagulation markers												
ln(CRP), 1 SD									-61 (44)	0.17	-65 (44)	0.14
Fibrinogen, 1 SD									41 (46)	0.37	44 (46)	0.34
No. of diagnosed chronic health conditions, 1 point											33 (42)	0.43

*Unadjusted. The difference in base pairs in mean leukocyte telomere length given a 1 standard deviation higher continuous covariate or the presence of a categorical covariate.

[†]Adjusted for age, gender, and race.

[‡]Additionally adjusted for education, body mass index, and current smoking status.

[§]Additionally adjusted for ln(CRP) and fibrinogen.

[‖]Additionally adjusted for number of diagnosed chronic health conditions.

Table 4.7.3: Association of leukocyte telomere length to disease in components of the physiologic index

Component of the Physiologic Index*	Mean (SD) LTL (bp)	Pearson r†	P†	Linear β (SE)‡	P‡
Carotid Artery Thickness					
0 (0.60-1.06 mm)	6540 (602)				
1 (1.06-1.53 mm)	6252 (591)	-0.214	0.001	-100 (39)	0.01
2 (1.53-3.94 mm)	6168 (600)				
White Matter Grade					
0 (0-1 units)	6422 (607)				
1 (2 units)	6386 (644)	-0.180	<0.01	-71 (41)	0.09
2 (3-9 units)	6121 (554)				
Serum Fasting Glucose					
0 (<100 mg/dL)	6365 (618)				
1 (100-126 mg/dL)	6256 (632)	-0.063	0.33	-49 (39)	0.21
2 (>126 mg/dL)	6239 (560)				
Serum Cystatin-C					
0 (0.6-1.0 mg/L)	6413 (651)				
1 (1.0-1.1 mg/L)	6344 (578)	-0.084	0.20	1 (41)	0.98
2 (1.1-3.5 mg/L)	6187 (606)				
Forced Vital Capacity					
0 (W: 2.6-3.8 L, M: 3.9-6.5 L)	6369 (592)				
1 (W: 2.2-2.6 L, M: 3.2-3.9L)	6352 (635)	-0.019	0.77	11 (52)	0.83
2 (W: 0.6-2.2 L, M: 0.3-3.2 L)	6218 (612)				

*Zero represents a normal physiologic value. Two represents higher disease burden.

†Correlation of leukocyte telomere length with disease in each component of the index, modeled as a continuous variable.

‡The difference in base pairs in mean leukocyte telomere length given a standard deviation higher component of the physiologic index, adjusted for age, gender, and race. Units are: carotid artery thickness, 0.49 mm; white matter grade, 1.4 units; serum fasting glucose, 28.9 mg/dL; serum cystatin-C, 0.30 mg/L; forced vital capacity, 0.89 L.

4.8 FIGURES

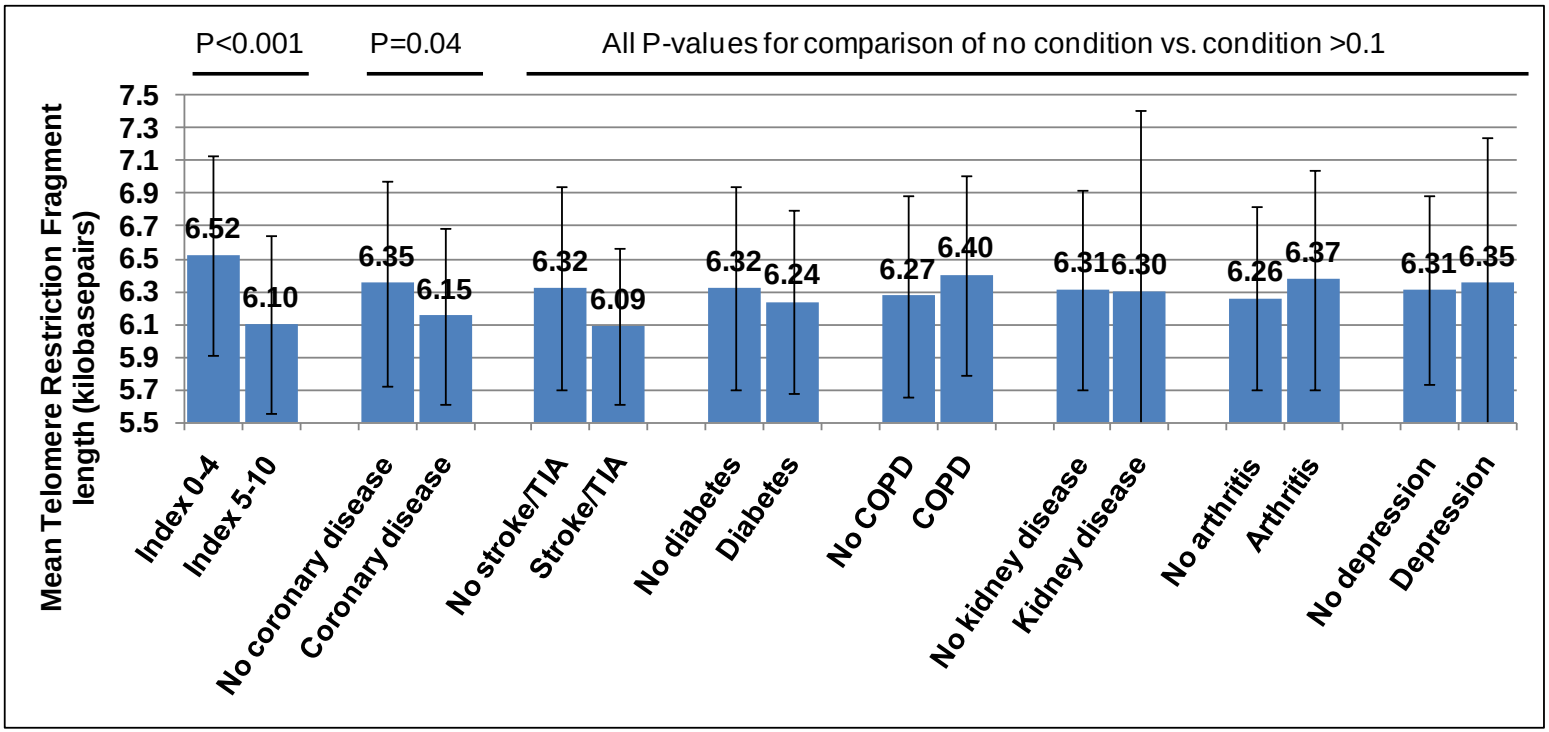

Figure 4.8.1: Mean (SD) LTL by index score category or presence of diagnosed chronic conditions

5.0 PAPER 2: THE ASSOCIATION OF CATARACT WITH LEUKOCYTE TELOMERE LENGTH IN OLDER ADULTS: DEFINING A NEW MARKER OF AGING

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5.1 ABSTRACT

Lens transparency, or the magnitude of cataract severity, is a potential *in vivo* marker of aging distinguishable from diagnosed cataract. To explore lens transparency as a marker of aging we determined its association with leukocyte telomere length (LTL), measured with qPCR. Cataract severity was directly measured in 259 participants and prevalent cataract and incident cataract surgery were ascertained in 2,750 participants of the Health, Aging, and Body Composition Study. LTL was unassociated with clinical cataract outcomes. Six of 259 had successfully aged lenses and a mean LTL of 5,700 bp while 253/259 with poorly aged lenses had a mean LTL of 4,770 bp. Participants with a 1,000 bp greater mean LTL had nearly half the odds of any cataract (OR=0.47, 95% CI 0.22-1.02) after adjustment. Lens transparency might be associated with longer LTL in community-dwelling older adults and should be investigated further as a possible biomarker of aging.

5.2 BACKGROUND

Individual health is highly variable. During their lifetimes, some individuals experience disease, accidents, and poor health; others seemingly escape these burdens. Underlying this variability is a single process that all living things experience: aging. It is critical to determine how we age if we hope to optimize the wellbeing of individuals. Specifically, markers of primary aging are needed as intermediate outcomes to understand the aging process and potential early benefits of preventive interventions.

The human lens is a marker of interest for several reasons. Lens changes, such as greater thickness and reduced transparency, can be detected *in vivo* in young adulthood, prior to the onset of disease and disability, and can be distinguished from known diseases of the lens such as cataract.[137,173-175] Lens proteins (crystallins) are set down in fetal development and are the only proteins in the body that do not turn over during life. Thus changes in lens proteins due to aging are not obscured by removal or repair processes and may reflect lifelong exposure. Furthermore, the same tissue can be repeatedly measured. Crystallins are natively clear proteins that belong to the family of chaperone proteins which critically maintain protein conformation, particularly under stress, throughout the body.[115-117] Disruption of lens crystallins, which leads to decreased transparency, may therefore reflect widespread breakdown of maintenance machinery, a process that underlies aging across tissues.[116,117] Indeed, the absence of crystallins that are normally found in the lens, brain, heart, skin, and skeletal muscle is associated with aging phenotypes.[121,122,176] Because crystallins are natively clear, lens transparency can also be

measured quickly, accurately, non-invasively and non-hazardously.[137,142,177] These qualities make lens transparency an attractive candidate marker of aging, though it has been nearly uninvestigated in population aging research.

To explore lens transparency as a marker of aging requires determining its association with other potential aging markers. Leukocyte telomere length (LTL), which shortens with age, inflammation, and oxidation, has become an increasingly prominent marker of aging.[25] It has been associated with longevity and some age-related outcomes independent of chronic disease in some, but not all, community-dwelling cohorts examined.[26,95,178,179] Subsequently, LTL could be a useful marker against which to correlate lens transparency. The hypothesis that lens transparency may be associated with LTL is also supported by the longstanding observation that the vast majority of individuals with the premature aging disorder Werner syndrome, which is characterized by accelerated telomere shortening, have early onset of bilateral cataracts.[180,181]

In this analysis we sought to explore the human lens as a possible marker of aging by studying the association of cataract with LTL in a large cohort of community-dwelling older adults, the Health, Aging and Body Composition Study (Health ABC), and an ophthalmic sub-study of Health ABC, the Age-Related Maculopathy Ancillary Study (ARMA). In doing so, we distinguish lens transparency, a possible primary aging phenotype, from cataract, a disease phenotype. We specifically hypothesized that 1) longer LTL is associated with a lower prevalence of cataract; 2) longer LTL is associated with a lower incidence of cataract surgery; and 3) longer LTL is associated with a lower odds of lens opacity.

5.3 RESEARCH DESIGN AND METHODS

5.3.1: Health ABC study population

We used the Health ABC study population to examine prevalence of cataract and incident cataract surgery. All Medicare-eligible people in Memphis, TN and Pittsburgh, PA were identified to volunteer in the Health ABC study. The study also sought an enriched sample of African-Americans. Because the Health ABC study was originally designed to examine incident mobility disability, eligibility criteria were no reported difficulty in walking for 1/4 mile, walking up 10 steps, getting in and out of bed or chairs, bathing or showering, dressing, or eating; no need of using a cane, walker, crutches, or other special equipment to get around; not enrolled in a lifestyle intervention trial; free of life-threatening illness; and had plans to stay in the geographic area for ≥3 years. Due to these criteria the study population is slightly healthier than the age-matched general population. The University of Pittsburgh and the University of Tennessee Institutional Review Boards approved all procedures related to Health ABC, and all research adhered to the Declaration of Helsinki. For the cross-sectional analysis of the association of baseline cataract diagnosis with LTL, the cohort included 2,750 Health ABC participants (41% black, 48% male, aged 70-79) who had available LTL measurements (89.4% of the original Health ABC cohort). The prospective analysis of incident cataract surgery included all those who did not report a diagnosis of cataract in either eye at baseline (N=1,505).

5.3.2: ARMA study population

We used the ARMA study, an ophthalmic sub-study within Health ABC, to examine lens transparency. The ARMA study included sensitive measures of lens transparency and recruited subjects from the Health ABC site in Memphis, TN to take part in a study of the role of carotenoids and inflammation in macular aging and the pathogenesis of age-related maculopathy. ARMA participants consisted of an enriched sample of subjects originally enrolled in the Health ABC study examined between Year 6 and Year 7 of the Health ABC study. Recruitment was based on stratification by a cumulative vision score from the Health ABC Year 3 tests (i.e., based on the outcome of binocular best corrected visual acuity to Bailey-Lovie charts, binocular contrast sensitivity measured with Pelli-Robson charts, and stereoacuity characterized with the Frisby test).[182] Inclusion criteria were non-random and included: self-reported diagnosis of age-related macular degeneration (AMD) (N=55); best vision function score (N=100); or worst vision function score (N=200). Exclusion criteria were: diagnosis of type 1 or type 2 diabetes of ≥6 years duration or presence of diabetic retinopathy at the time of the eye exam; known history of glaucoma, or documented presence thereof on examination; diagnosis of autoimmune disease or monoclonal gammopathy; illiteracy based on the inability to perform visual acuity tests, which negated reliable calculation of vision score, unless the patient was referred for diagnosis of AMD. Our cross-sectional analysis of lens transparency included all 259 Health ABC participants who had their mean LTL determined at baseline and who also had their lens transparency measured as part of the ARMA study. Though ARMA participants were non-randomly selected from the Health ABC study Memphis cohort using vision scores, selection was unassociated with baseline mean LTL ($p=0.76$), age, gender, smoking history, BMI, total cholesterol, and history of hypertension, heart disease, or cancer (all $p>0.05$). However, ARMA

participants were less likely to be black, more likely to use statins, and had lower mean HDL, oxidized LDL (oxLDL), C-reactive protein (CRP), interleukin-6 (IL-6), and fasting glucose (all $p<0.05$), which was expected given the ARMA selection criteria.

5.3.3: Cataract ascertainment and lens transparency measures

At the baseline Health ABC clinic visit, trained interviewers asked subjects if a physician ever told them they had a cataract in one eye (unilateral cataract) or both eyes (bilateral cataract). We analyzed unilateral and bilateral cataract independently as measures of prevalent baseline cataract. Because retrospective determination of cataract diagnosis could introduce recall bias, we also analyzed incident cataract surgery documented prospectively by trained interviewers at annual clinic visits and at 6 month intervals with a home, phone, or proxy questionnaire.

Screening for ARMA included a dilated anterior segment exam by one observer (A.I.) to verify the presence of the crystalline lens and to ascertain the presence of cataract (nuclear, cortical, or both). Lens transparency was graded according to the validated Age-Related Eye Disease Study (AREDS) criteria.[143] AREDS grading was accomplished by comparing an individual's lens transparency measured using a slit lamp at the time of examination to standard, graded photographs. The AREDS grading system for nuclear cataract is a semi-continuous, 8-step based scale, with 1 indicating complete transparency and 8 indicating complete cataract, and where opacity of intermediate severity between steps can be classified by 0.5 intervals. The AREDS scale for anterior cortical and posterior cortical cataract is measured as the percent of the viewing field occupied by the opacity (0-100%).

5.3.4: Telomere measurement

DNA was extracted from isolated peripheral blood mononuclear leukocytes. Average LTL was measured using a validated qPCR method, as described previously.[104] This method measures the relative average LTL in genomic DNA by determining the ratio of telomere repeat copy number to single copy gene copy number (T/S ratio) in experimental samples relative to a reference sample. The coefficient of variation for the relative T/S ratios was 5.8%. Assuming a normal distribution for relative T/S ratios in repeated measurements of the same sample, samples differing in average LTL by as little as 11.4% should be distinguishable by this method at the 95% confidence level. A T/S "ratio unit" of 1.0 measured by the qPCR method is equivalent to a mean LTL of 4,270 base pairs, the known LTL of the reference DNA used in this study. Thus, the LTL unit presented was converted to base pairs by using this conversion factor.

5.3.5: Covariates

Trained interviewers administered the baseline Health ABC questionnaire between April 1997 and May 1998 to assess demographic and socioeconomic characteristics, health behaviors, health status, and medical history. Within 2 weeks of the interview subjects visited the University of Pittsburgh or University of Tennessee clinics for baseline biologic, anthropometric, and functional measures and a blood draw. We selected possible confounders measured at baseline based on those documented in the literature. Demographic characteristics included age, gender, race (white or black), and study site. Smoking was categorized as never, former, or current. Body mass index (BMI, kg/m^2) was calculated using baseline height and weight. The prevalence of physician-diagnosed diabetes, ischemic heart disease, congestive heart failure, cerebrovascular disease, and cancer (excluding non-melanoma skin cancer) was determined using algorithms

based on self-reporting and medication use. Diabetes was defined as an elevated fasting glucose ($\geq$126 mg/dL) or abnormal 2-hour oral glucose tolerance test ($\geq$200 mg/dL). Blood pressure was measured with the participant seated for 5 minutes and then one minute after standing. Participants were asked to bring all prescription and over the counter medications used in the previous two weeks. Medications were coded using the Iowa Drug Information System.[183] Assay reproducibility was checked using a blind duplicate system whereby 5% of aliquots had a duplicate aliquot prepared. Blind duplicate aliquots were analyzed simultaneously and results were matched to determine the Pearson correlation coefficient and coefficient of variation.

5.3.6: Statistical analysis

We assessed whether covariates could be potential confounders by quantifying their association with LTL, cataract, cataract surgery, or lens transparency using Pearson correlation coefficients, the student's T-test, χ^2 statistic and analysis of variance. We chose the significance level $p<0.10$ as a cutoff for inclusion of possible confounders in multivariate models. Age, BMI, cholesterol, HDL, LDL, oxLDL, CRP, IL-6, fasting insulin, and fasting glucose were treated as continuous covariates while gender, race, study site, smoking (ever/never), comorbidities, and medication use were treated categorically. oxLDL, CRP, IL-6, and fasting insulin levels were log-transformed in models to account for skewness, though non-transformed descriptive statistics are reported.

We added LTL to all models in kilobasepairs such that ratio measures reflect the odds of outcome associated with a 1,000 bp increase in mean LTL. LTL was measured on 45 qPCR plates so we included a random effect variable in all models to account for slight LTL measurement variation between plates. For analyses of cataract in Health ABC, we used logistic

regression to generate odds ratios and 95% confidence intervals to determine the association between LTL (predictor) and baseline cataract (outcome). We employed multivariate logistic regression for adjustment. We used a frailty model for Cox proportional hazards regression to calculate the hazard ratio for incident cataract surgery (outcome) using LTL as the predictor. Multivariate proportional hazards regression was used to adjust for confounding.

Because we were interested in exploring the lens as a marker of successful aging, in the ARMA study, we decided *a priori* upon a stringent classification of a truly transparent lens to clearly distinguish successful lens aging from cataract. Using the AREDS grading criteria, we defined successful lens aging as ≤3.0 nuclear grade and 0% cortical grade. An aged lens was defined as having nuclear opacity >3.0 grade or cortical opacity >0% grade or previous placement of an intraocular lens (N=102). Specific types of cortical opacity were also classified using the AREDS criteria. The reference group for all logistic models of lens transparency was participants with a transparent lens (≤3.0 nuclear grade and 0% cortical grade, N=6). We used logistic regression and multivariate logistic regression for all ARMA analyses.

We simultaneously tested for interaction between LTL and all included covariates using a significance level of 0.10. Non-significant interactions were excluded from the final multivariate models. We used a significance level of 0.05 except as noted earlier. SAS 9.1 (SAS Institute, Cary, NC) was used for all analyses except proportional hazards regression (Stata 10.0, StataCorp, College Station, TX).

5.4 RESULTS

Prevalent cataract

Mean LTL at baseline for all participants was 4,860 bp (SD, 1,360 bp). Though correlations were weak (all $r<0.103$), LTL was shorter with greater age, BMI, oxLDL, IL-6, fasting glucose, and fasting insulin, and longer with greater HDL and total cholesterol (all $p<0.10$). At baseline, mean LTL was 377 bp greater in women ($p<0.0001$), 107 bp greater in Blacks ($p=0.043$), and 131 bp greater in statin users ($p=0.016$). Smoking history was associated with shorter LTL ($p=0.0008$).

At baseline, 36.4% and 25.6% of participants reported a history of unilateral and bilateral cataract, respectively. Both unilateral and bilateral cataract was significantly associated with age and gender (Table 5.7.1, bilateral cataract data not shown). Participants reporting a history of unilateral cataract had greater BMI, were more likely to be from Pittsburgh, and had a greater burden of comorbidities. Crude analyses illustrated no association between LTL and unilateral or bilateral cataract (Table 5.7.2). After adjustment for relevant confounders, the magnitude of effect increased slightly but remained non-significant.

Incident cataract surgery

Over nine years of follow-up, 353 of 1,505 participants underwent cataract surgery for an average nine-year risk of 23.5% and a rate of 17.2/1,000 person-years. The average length of follow-up was 8.1 years. Incident cataract surgery was associated with age, gender, cholesterol,

study site, and statin use (Table 5.7.1). LTL was not associated with nine-year risk of cataract surgery before or after adjustment (aHR=1.02, 95% CI 0.94-1.10) (Table 5.7.2).

Lens transparency

Mean LTL at baseline for all ARMA participants was 4,790 bp (SD, 1,050 bp). At the time of the ARMA study eye exam, 97.7% of participants had evidence of any cataract, including 95.0% with nuclear opacity (AREDS grade >3.0), 79.2% with anterior cortical opacity, and 52.5% with posterior cortical opacity. The 6 individuals with successfully aged lenses (nuclear grade ≤3.0 and 0% cortical grade) had a mean LTL of 5,700 bp and no history of smoking, cerebrovascular disease or cardiovascular disease. For each 1,000 bp increase in mean LTL, subjects were nearly 50% less likely to have any cataract or an intraocular lens (i.e. an aged lens) (Table 5.7.3). There was a similar protective effect for nuclear opacity, any cortical opacity and cortical opacity subtypes. Adjustment for confounders included in the previous cataract models did not alter estimates appreciably.

5.5 DISCUSSION

This is the first report of the association between cataract and LTL in human subjects. Using a stringent *a priori*-determined cutoff to define successful lens aging, it is striking that we identified individuals with a mean LTL nearly 1,000 bp longer than other individuals with less transparent lenses. Our analysis suggests that higher lens transparency was likely associated with longer LTL. Both transparency and LTL may indicate cumulative oxidation.[25,184] Alternatively, senescence of the lens epithelium has been associated with non-enzymatic alterations in lens crystallins and this may correlate with senescence-related immune aging. In contrast, diagnosed cataract, a clinical disease phenotype, was not associated with LTL. This could be because self-reported physician diagnosis of cataract is a biased measure, because cataract surgery is more dependent on access to care or impact on quality of life than the severity of the cataract, or because diagnosed cataract is a marker of disease and not necessarily aging. Cataractous lenses are different from aged human lenses in gene expression patterns,[138] and the clinical classification of "cataract" or "no cataract" does not reflect the subtle morphological, histological, and biochemical changes that occur with lens aging.[137,139-141] These data provide preliminary evidence that lens transparency might be an *in vivo* marker of primary aging whereas diagnosed cataract is likely not. With further exploration, lens transparency might emerge as a valuable intermediate marker for use in intervention studies attempting to modify the aging process.

Using our stringent definition of transparency, we identified only 6 individuals free of opacity in the ARMA sub-study. Subsequently, this definition illustrates a truly rare phenotype of successful aging. This rarity is akin to using the oldest old, such as centenarians and super-centenarians, to search for longevity-associated factors, yet may be more feasible because the phenotype can be identified at younger ages. Interestingly, at the Health ABC baseline clinic visit, these 6 participants had no history of smoking, cerebrovascular disease or cardiovascular disease in addition to markedly longer LTL, yet their mean age was 72.3 years (range 70-75). This is further proof that sensitive lens measurements may provide a window on whole organism aging.

The lens has several real and hypothesized advantages as a marker of aging. First, the lens is unique in that nuclear lens cells are present at birth and do not turn over throughout life. Therefore, the lens has the potential to reflect risk factors for aging that are encountered throughout life, and the same tissue can be measured repeatedly. Second, though a somewhat immune privileged site, the lens is exposed to the internal and external environments. Subsequently, transparency might reflect both *in vivo* and *ex vivo* risk factors for aging. Third, using modern imaging techniques, transparency can be measured on a continuous scale, allowing finer risk stratification. Fourth, lens imaging is non-invasive, non-hazardous, and quick, so it may be easily adopted by researchers and clinicians. The utilization of a grading system, such as the AREDS one applied here[143] or the LOCS-III or the OCCCGS systems,[144,145] offers a simple, fast, inexpensive, reproducible, and accurate means to ascertain lens transparency. Although AREDS grading is designed to be applied by trained graders after the eye exam on standard photos obtained with a photographic slit lamp, adding to the complexity and duration of the eye exam and significantly increasing cost, it surely offers an accurate and minimally biased

assessment of lens transparency. Other imaging techniques, such as Scheimpflug photography, can provide a more accurate assessment of the nucleus, can be acquired quickly, and allow finer quantification of transparency, though it can be expensive and requires training.

Our results are strengthened by several factors. This study represents one of the largest samples of LTL measurements available in prospective longitudinal studies, a necessity given the inherent heterogeneity in human LTL.[100] Coupled with measurement of lens transparency and LTL using validated methods,[104,143] low loss-to-follow-up and long average length of follow-up, we believe our estimates are minimally affected by random error and bias. Similar to previous studies, we also found that LTL was greater in women, blacks, non-smokers,[64] and those without cardiovascular disease.[57,75,87] The adjusted odds ratios for covariates included in our multivariate models also agree with those previously published,[185-188] suggesting our models are accurate because they are consistent with those from other populations. Though the ARMA sample was small, which limited power, adjustment for other confounders did not alter the magnitude of estimates appreciably.

Our study does have some limitations. Although we determined that the ARMA population was relatively similar to the overall Health ABC cohort at baseline, selection of the ARMA study population was non-random with respect to outcome, which could introduce selection bias. It is possible that residual confounding affects our final estimates due to incomplete adjustment, as well as possible inaccuracies in lens transparency estimation introduced by the AREDS grading system. Although we found no significant interactions, unidentified modification might remain. Finally, we did not examine associations with posterior subcapsular cataract, though this is a rare lesion.[185-187]

In conclusion, we found that greater lens transparency is likely associated with longer LTL in a sample of community-dwelling older adults. This suggests that lens transparency might serve as an *in vivo* marker of primary aging. We recommend that future studies of this association employ a prospective approach to more accurately portray the biological dynamic which occurs, namely studying telomere attrition and declining transparency using repeated measures. This would also mirror how these markers might be employed in the future to monitor biologic age across the lifespan. Because lens transparency decreases beginning in adolescence and LTL is heterogeneous at birth, it would be most sensible to investigate whether the rate of telomere shortening is associated with rate of change in lens transparency in a study of participants with a wide age range. Finally, testing whether lens transparency is a predictor of mortality or longevity may provide the strongest evidence of its usefulness as a biomarker of aging.

5.6 ACKNOWLEDGEMENTS

Authors' contributions: conception and design (JLS, AI, ABN); acquisition of data (AI, WCH, SRC, RMC, TBH, ABN); analysis and interpretation of data (JLS, AI, WCH, RMB, RMC, MAN, ABN); drafting of the manuscript (JLS, ABN); critical revision of the manuscript (JLS, RMB, YPC, PLO, SRC, RMC, TBH, MAN, SBK, ABN); statistical analysis (JLS, RMB); obtaining funding (SRC, ABN); administrative, technical, or materials support (ABN); supervision (ABN).

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5.7 TABLES

Table 5.7.1: Baseline characteristics of the Health ABC Study population by prevalent cataract at baseline or incident cataract surgery

	Unilateral cataract			9 yr cataract surgery		
	Yes (N=984)	No (N=1,719)		Yes (N=353)	No (N=1,152)	
	Mean (SD) or N (%)	Mean (SD) or N (%)	*P**	Mean (SD) or N (%)	Mean (SD) or N (%)	*P**
Age (years)	74.1 (2.9)	73.4 (2.8)	<0.0001	73.5 (2.8)	73.1 (2.7)	0.05
Gender						
Male	428 (43.5)	880 (51.2)	0.0001	157 (44.5)	652 (56.6)	<0.0001
Female	556 (56.5)	839 (48.8)		196 (55.5)	500 (43.4)	
Race						
White	584 (59.4)	1014 (59.0)	0.85	215 (60.9)	652 (56.6)	0.15
Black	400 (40.6)	705 (41.0)		138 (39.1)	500 (43.4)	
Study site						
Memphis	466 (47.4)	905 (52.6)	0.008	153 (43.3)	599 (52.0)	0.004
Pittsburgh	518 (52.6)	814 (47.4)		200 (56.7)	553 (48.0)	
Smoking status						
Never	418 (42.5)	752 (43.9)	0.022	161 (45.6)	490 (42.7)	0.14
Current	83 (8.4)	194 (11.3)		30 (8.5)	141 (12.3)	
Former	482 (49.0)	769 (44.8)		166 (45.9)	517 (45.0)	
Comorbidities						
Diabetes mellitus	173 (17.6)	229 (13.4)	0.003	58 (16.5)	144 (12.6)	0.06
Hypertension	397 (40.4)	655 (38.1)	0.46	133 (37.7)	450 (39.1)	0.87
Cerebrovascular disease	88 (9.1)	99 (5.9)	0.002	17 (4.9)	74 (6.5)	0.27
History of MI	24 (2.5)	51 (3.0)	0.42	11 (3.2)	34 (3.0)	0.88
History of cancer	190 (19.4)	272 (15.9)	0.023	60 (17.1)	176 (15.4)	0.46
Current statin use	123 (12.5)	231 (13.5)	0.46	60 (17.1)	147 (12.8)	0.044

*P-value from T-test, ANOVA, or χ^2-test.

Table 5.7.2: Models of lens disease: likelihood of cataract and cataract surgery with each 1,000 basepair increase in leukocyte telomere length

Cataract Outcome	Prevalence or Rate	Crude OR/HR	Crude 95% CI	Adjusted OR/HR*	Adjusted 95% CI*
Baseline unilateral cataract	36.4%	0.95	0.89-1.01	0.95	0.89-1.01
Baseline bilateral cataract	25.6%	0.99	0.93-1.06	0.99	0.92-1.06
9yr incident cataract surgery	17.2/1000py	1.03	0.96-1.12	1.02	0.94-1.10

CI, confidence interval. HR, hazards ratio. OR, odds ratio.
*All adjusted for age, gender, site, smoking, BMI, HDL, cholesterol, and statin use.

Table 5.7.3: Models of successful lens aging: odds of lens opacity vs. no opacity with each 1,000 basepair increase in leukocyte telomere length

Opacity Outcome	N	Mean (SD) LTL (bp)	OR (95% CI)	OR (95% CI)*
No opacity	6	5,700 (1,550)	Ref	Ref
Nuclear opacity	246	4,780 (1,040)	0.51 (0.27-1.00)	0.48 (0.22-1.03)
Anterior cortical opacity	205	4,790 (1,040)	0.52 (0.27-1.01)	0.50 (0.23-1.05)
Posterior cortical opacity	136	4,760 (1,050)	0.50 (0.24-1.02)	0.47 (0.21-1.05)
Any cortical opacity	214	4,800 (1,040)	0.52 (0.27-1.01)	0.49 (0.23-1.05)
Any opacity or IOL	253	4,770 (1,040)	0.51 (0.26-0.99)	0.47 (0.22-1.02)

CI, confidence interval. OR, odds ratio.

*Adjusted for age and gender.

6.0 PAPER 3: IS LENS TRANSPARENCY ASSOCIATED WITH CARDIOVASCULAR OR METABOLIC DISEASE OR COGNITIVE FUNCTION IN OLDER WOMEN? DEFINING A NEW BIOMARKER OF AGING

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6.1 ABSTRACT

Background: Few primary markers of aging are validated in human population studies. The lens is of interest because it remains from birth until death and can be measured accurately and noninvasively. To evaluate if the lens is an indicator of aging, disease, both, or neither, we determined the association of lens transparency and cataract surgery to markers of aging and disease including risk factors for coronary atherosclerosis, coronary artery calcium (CAC), aortic calcium (AoC), carotid intima-media thickness (IMT), fasting plasma glucose or insulin, cognitive function and ApoE genotype.

Methods: We directly measured lens transparency with Scheimpflug photography (N=136) and enumerated self-report of cataract surgery (N=231) in participants of the Healthy Women Study (mean (SD) age 73.3 (1.7) years). Atherosclerosis risk factors included age, smoking, waist circumference, BMI, lipids, blood pressure, fasting glucose and insulin, interleukin-6, and medication use and were measured concurrently with lens transparency and 25 years previously. CAC and AoC were measured with electron beam tomography at the time of lens measurements and ~7, 10, and 13 years previously. IMT was measured with ultrasound. Cognition was measured with the Modified Mini Mental Status exam.

Results: Cross-sectionally, cataract surgery was associated with larger waist circumference and diabetes ($P<0.05$). Lens transparency <95% vs. transparency ≥95% was associated with diabetes

(P<0.05). Cataract surgery was associated with greater baseline BMI and higher baseline triglycerides. Participants with transparency <95% had higher baseline insulin. Cataract surgery or lens transparency was not associated with CAC, AoC, or IMT. Cataract surgery was associated with lower median 3MS score. Participants with less transparent lenses were more likely to harbor an ApoE 3/4 genotype.

Conclusions: Low lens transparency and cataract surgery are associated with diabetes. Low lens transparency is associated with higher frequency of ApoE 3/4 genotype, but not with risk factors for atherosclerosis, CAC, AoC, or IMT. Replication in cross-sectional studies in different populations could help demonstrate if lens transparency is a noninvasive indicator of brain pathology and cardiovascular health.

6.2 BACKGROUND

Few primary markers of aging have been validated in human population studies. With an increasing emphasis on lifecourse epidemiology, which leverages longitudinal analysis to examine processes with long incubation periods, such as aging, there is a need to uncover predictive markers which could be measured repeatedly over long periods of time. It would be ideal to define noninvasive tests of change in tissues that do not turn over because these markers may provide insight on the integrity of a human throughout the lifespan.

The human lens is of interest because it develops *in utero* and central lens cells (the lens nucleus) remain from birth until death. Subsequently, in theory, the lens may provide a record of lifetime exposure to risk factors for accelerated aging and/or disease. The lens is freely visible and comprised of natively clear proteins named crystallins, allowing it to be measured simply and noninvasively with imaging. Furthermore, the biology of the lens suggests it may provide insight on tissue integrity throughout the body. Lens crystallins belong to the family of chaperone proteins which maintain protein conformation, particularly under stress.[115-117] Perturbation of lens crystallins, which leads to decreased transparency manifested clinically as cataract, may indicate breakdown of maintenance machinery, a form of failing stress response, which is a process underlying aging.[116,117] Lens transparency, a continuous measurement, can also be differentiated from a disease phenotype, such as age-related cataract, and thus may serve as a more sensitive marker than dichotomous cataract status. Currently it is unknown if the lens

is a valid biomarker of aging in humans because it has not been examined in association with markers of aging and disease.

Some of the most common and burdensome age-related diseases are cardiovascular disease, diabetes mellitus, and dementia. Subsequently, examining the association of the lens to markers of these diseases will provide evidence for the ability of the lens to reflect aging or age-related disease. Several recent reports from large, longitudinal studies document the strong association between coronary artery calcification (CAC) and risk of coronary heart disease and death.[189-191] CAC is now accepted as a powerful marker of coronary heart disease and predictor of cardiovascular disease and death independent of other risk factors for cardiovascular disease, such as smoking, lipid levels, blood pressure, waist circumference, and inflammation. Fasting glucose and insulin insensitivity are used to define diabetes. They are thus prime measures of metabolic health. The Modified Mini Mental Status Exam (3MS) is a marker of cognition used clinically to detect dementia. Additionally, Alzheimer's-type dementia is strongly associated with the apolipoprotein E4 (ApoE4) genotype, a genetic marker.

In this analysis we evaluate the association of lens transparency and cataract surgery to markers of aging and disease, including: 1) risk factors for coronary atherosclerosis, the extent of noninvasively measured coronary atherosclerosis, fasting plasma glucose or insulin; 2) cognitive function measured with the 3MS and ApoE genotype. We utilize a cross-sectional study of the association of current measures of aging and disease to lens transparency and history of cataract surgery. Furthermore, to more carefully investigate temporality, we conduct a retrospective longitudinal analysis by testing the association of previous measures of aging and disease to current lens transparency or history of cataract surgery. This analysis will help clarify if the lens may be a valid indicator of aging and/or age-related chronic disease.

6.3 RESEARCH DESIGN AND METHODS

6.3.1: Study population

We conducted lens measurements in participants of the Healthy Women Study (HWS). The HWS is a community-based longitudinal cohort study that began in 1983-1984 in Pittsburgh, PA, selecting a sample of 541 women (mean (SD) age 47.5 (1.6) years), all premenopausal, who did not have diabetes or clinical hypertension. The HWS was the first study to determine changes in risk factors from the pre- to the postmenopausal period and possible relationship to changes in endogenous hormone levels. Women were followed yearly by mail and telephone follow-up and had several clinical examinations. The study pioneered in the evaluation of measures of subclinical atherosclerosis including the evaluation of coronary and aortic calcification by electron beam tomography (EBT).

In the current HWS visit cycle, participants underwent a fourth EBT scan of CAC 25 years after the original enrollment, returning when they were 67-79 years old. A blood sample was also stored for molecular and genetic study. Approximately 80% of the 289 women that would have had their third EBT, i.e., a total of 231, were predicted to return for their fourth measurement of CAC. We measured lens transparency when women returned for their fourth EBT scan. Data on the lens was obtained for 231 women. The HWS and Aging Lens Sub-study were approved by the University of Pittsburgh Institutional Review Board. All participants gave consent for the studies.

6.3.2: Lens measurements

At their fourth EBT visit, women were asked about their history of cataract surgery or diagnosis of glaucoma, macular degeneration, eye trauma, or other eye diseases (N=231). Women who had bilateral cataract surgery (N=64, 27.7%) or those with a history of glaucoma (N=22, 9.5%) who are at risk for acute narrow angle glaucoma with dilation were ineligible for lens transparency measurement, leaving 145 women eligible for direct measurement of lens transparency. Thirty eight women (16.5%) were not interested and 35 women (15.1%) were unavailable for direct lens measurement, but did provide data on history of cataract surgery. Participants were unavailable due to an inability to return to the clinic for the lens measurements due to scheduling conflicts, caregiver responsibilities, or living too far from Pittsburgh. Subsequently, we measured lens transparency in 68 right eyes and 72 left eyes (4 participants had undergone cataract surgery in only the right eye).

Before dilation, participants' best corrected visual acuity, contrast sensitivity, and depth perception were measured using the Bailey-Lovie distance visual acuity test, Pelli-Robson contrast sensitivity test, and Frisby stereo test, respectively.[182] Lens transparency was measured during an eye exam where both pupils were dilated using proparacaine hydrochloride ophthalmic solution 0.5% (Ophthetic©, an anesthetic), phenylephrine hydrochloride ophthalmic solution 2.5% (Mydfrin©), and tropicamide ophthalmic solution, USP 1% (Mydriacyl©). The lens exam was performed approximately 10-20 minutes after dilation drops were administered and required approximately 10 minutes. Lens transparency was obtained using Scheimpflug photography.[137,142,146] For Scheimpflug imaging, three to four retroillumination images of each intact lens were taken at equal intervals from 90^{O} to -90^{O} for each participant. The optical measurements were averaged to give an average quantitative measurement of total lens

transparency for each eye. A Nidek EAS-1000 anterior eye segment analysis system was used. This system consists of a slit lamp connected to a CCD camera whose position is moved by an electric motor based on preset conditions. All photographic and optical density measurements were stored in a computer for retrieval and analysis.

6.3.3: Measurement of risk factors for coronary atherosclerosis, coronary artery calcification, fasting plasma glucose or insulin

Risk factors for coronary atherosclerosis included age, smoking (determined from self report), waist circumference and body mass index (determined by anthropometric measurement), total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides (all measured with NMR spectroscopy), blood pressure (determined with sphigmomanometry using a standard protocol), medication for altering lipids or blood pressure, and interleukin-6 (IL-6, measured with an enzyme-linked immunosorbent assay), and marker of inflammation that increases with age.[192] Risk factors measured at the fourth EBT examination were used for cross-sectional analysis and risk factors measured at the baseline HWS examination (~25 years previously) were used for longitudinal analysis. Some variables recorded at the fourth EBT examination (e.g., waist circumference, use of lipid and blood pressure medications, IL-6) were unavailable at the baseline HWS examination. EBT scans for CAC were performed using a GE-Imatron C-150 (Ultrafast CT ®) scanner (Imatron, South San Francisco, CA). For evaluation of the coronary arteries, 30 to 40 contiguous 3 mm thick transverse images were obtained from the level of the aortic root to the apex of the heart. Images were obtained during maximal breath holding using ECT triggering so that each 100 millisecond exposure is obtained at 60% of the R-R interval. Calcium scores for the coronary arteries were

calculated using the method of Agatston, resulting in both a total score and a total number of calcifications, and more recently by volume scores. Calcification was considered present when at least 3 contiguous pixels of 130 Houndsfeld Units are present on a 30 cm matrix. Initial image analysis was performed on a DICOM workstation using software provided by AculmageInc. Spearman correlations between the Agatston scores and volumetric scores was 0.94 for the coronaries. The mean time between first and second EBT examination was 3.19 years. The mean time between the first and third EBT examination was 6.44 years. Spearman correlation between the 1st and 2nd CAC in the HWS was 0.84, between the 1st and 3rd was 0.84, and between the 2nd and 3rd was 0.86. Pearson correlations were similar in magnitude. CAC was classified as previously in the HWS: 0, 1-99, and ≥100. Fasting plasma glucose and insulin were measured from blood using standardized clinical lab procedures. Diabetes was defined as self-report of physician diagnosis, hypoglycemic medication use, or glucose level of 126 mg/dL or higher, as established by the American Diabetes Association.[167]

6.3.4: Measurement of cognition and ApoE genotype

Cognitive function was measured by trained interviewers using the 100-point Teng Modified Mini Mental Status Exam.[193] ApoE genotype was detected by isoelectric focusing followed by immunoblotting. HWS serum samples were delipidated using the guanidine-HCI method[194] followed by an isoelectric focusing/immunoblotting protocol.[195]

6.3.5: Statistical analysis

We assessed the association of bilateral cataract surgery (a theorized disease phenotype) or lens transparency (a theorized aging phenotype) to indicators of aging and age-related chronic

disease. For bilateral cataract surgery, participants were categorized into two groups: bilateral cataract surgery present (N=64) vs. bilateral cataract surgery absent (N=167). For lens transparency, participants were categorized into two groups based on two different cutpoints: ≥90% total transparency (N=39) vs. <90% total transparency or cataract surgery in that eye (N=97); ≥95% total transparency (N=26) vs. <95% total transparency or cataract surgery in that eye (N=110). These cutpoints were chosen to 1) establish phenotypes illustrative of successful lens aging (i.e., a healthy aging phenotype); 2) to help balance group size based on the distribution of total transparency, which was skewed because many participants had relatively transparent lenses or had undergone cataract surgery. To be categorized in the successful lens aging group (i.e., ≥90% transparency or ≥95% transparency), a participant's right and left eyes both had to have transparency above the cutpoint. If data were available for only one eye then that eye was used for categorization.

For cross-sectional analysis, we determined the association of lens transparency measured at the fourth EBT examination to markers of aging and disease measured at the fourth EBT examination. For retrospective longitudinal analysis, we determined the association of lens transparency measured at the fourth EBT examination to markers of aging and disease measured at the baseline HWS examination (25 years previously), and also to CAC measured at the first (13 years previously), second (10 years previously), and third (7 years previously) EBT examination. We used the two-sample t-test or χ^2-test to assess the association of cataract surgery/lens transparency to continuous and categorical variables, respectively. We also used the median one-way test to assess the association of cataract surgery/lens transparency to median IL-6 level, median CAC level, and median 3MS score. A P-value <0.05 was used to determine statistical significance. All analyses were conducted with SAS 9.2 (SAS Institute, Cary, NC).

6.4 RESULTS

At the fourth EBT examination participants were on average 73.3 (SD 1.7) years old. For their age, participants had a healthy profile: 98 (42.4%) had total cholesterol <200 mg/dL, 108 (46.8%) had a systolic blood pressure <120 mmHg, 214 (92.6%) had a fasting glucose <126 mg/dL, and only 2.6% were current smokers. Of note, 98 (42.4%) were on lipid-lowering therapy, 101 (43.7%) were on blood pressure-lowering therapy, and 16 (6.9%) were on diabetes therapy. Fifty five percent of participants with total cholesterol <200 mg/dL were on lipid therapy, 39% of participants with systolic blood pressure <120 mmHg were on blood pressure therapy, and 3% of participants with fasting glucose <126 mg/dL were on diabetes therapy.

Sixty four (27.7%) women had previously undergone bilateral cataract surgery, 22 (9.5%) had been diagnosed with glaucoma, and 8 (3.4%) had been diagnosed with other eye diseases (Table 6.7.1). Transparency in right and left eyes was correlated in the lens nucleus ($r = 0.641$, $P<0.0001$) and cortex ($r = 0.724$, $P<0.0001$), and for total transparency ($r = 0.735$, $P<0.0001$). Nuclear cataract (the percent of the nucleus scattering light) in left and right eyes ranged from 0%-15.5%. Cortical cataract ranged from 0%-67.1%, but was overall relatively low, with a median (interquartile range) of 3.7% (8.1%) in right eyes and 5.5% (12.3%) in left eyes. Total transparency was on average high (mean (SD) 91.9% (10.6%) in right eyes and 88.0% (15.3%) in left eyes). Participants' visual acuity, contrast sensitivity, and depth perception were overall in the healthy range (Table 6.7.1). There were 39/136 and 97/136 participants with transparency ≥90% and <90%, and 26/136 and 110/136 participants with transparency ≥95% and <95%.

Regarding risk factors for coronary atherosclerosis, cross-sectionally, cataract surgery was associated with larger waist circumference, use of diabetes medication and diabetes status by medication or glucose level (all $P<0.05$) (Table 6.7.2). Participants with lens transparency <95% were also more likely to be diabetic than participants with transparency ≥95%. Cataract surgery was associated with greater baseline BMI and higher baseline triglycerides, and participants with transparency <95% had higher baseline fasting insulin (Table 6.7.3). Although there was a trend that cataract surgery or lower lens transparency was associated with higher IL-6 level, this trend was non-significant. Lens measurements were not associated with other risk factors for coronary atherosclerosis either cross-sectionally or longitudinally.

An age-associated increase in mean CAC was clearly seen among HWS participants (Table 6.7.4). Cataract surgery or lens transparency was not associated with CAC level measured at the same time or previously (Table 6.7.4). Nonetheless, there appeared to be a non-significant trend that participants without cataract surgery or with greater transparency had lower CAC levels. In additional analyses we tested the association between the lens and EBT-measured total aortic calcium score (Table A.1.1), which increases with age and is more reflective of age-associated arteriosclerosis rather than atherosclerosis, and carotid intima-media thickness (Table A.1.2), which also increases with age but also with high blood pressure and atherosclerosis. Aortic calcium or carotid intima-media thickness were not associated with cataract surgery or lens transparency.

HWS participants had high median 3MS scores (Table 6.7.5), far above a score of 80 used clinically to denote dementia. Despite this ceiling effect, cataract surgery appeared to be associated with lower median 3MS score, though lens transparency was not associated with 3MS score. Overall, lens transparency was associated with ApoE genotype ($P<0.05$). As high lens

transparency was more stringently identified by the absence of cataract surgery, then ≥90% transparency, then ≥95% transparency, the frequency of an ApoE4 allele fell from 19.5% to 15.8% to 11.5%, which was driven by a lower prevalence of the 3/4 genotype.

6.5 DISCUSSION

In a population of relatively healthy older women, we found that cataract surgery or lens transparency was not associated with most examined risk factors for coronary atherosclerosis or CAC, aortic calcium, or carotid intima-media thickness measured concurrently or previously. There was a relatively consistent association between lower transparency and markers of diabetes. This was expected given the established relationship between diabetes and cataract. Finally, we observed that transparency was overall associated with ApoE genotype, and that greater transparency may be associated with a lower frequency of carrying an ApoE4 allele and better cognitive function.

The association between the lens and brain is intriguing. If true, it suggests the lens may be an indicator of brain aging or disease. Histological and biochemical studies support the hypothesis that the lens may reflect brain pathology. Alpha-B crystallin, a main component of the lens, is found in the brain,[121] particularly in the limbic and paralimbic regions, which are commonly affected in Alzheimer's patients.[126] The number of alpha-B crystallin positive neurons increases in parallel with neuronal loss.[126] Alpha-B crystallin is upregulated in astrocytes associated with senile plaques and cerebral amyloid angiopathy in Alzheimer's disease patients,[127] and it inhibits aggregation of A-beta peptide *in vitro*.[128] Recent evidence also suggests that ApoE exists in the lens capsule,[196] and that beta-amyloid toxically aggregates in the lens.[197,198] This data supports crystallin's role as a heat shock protein that maintains protein

conformation and responds to intracellular stress, and offers clear links between the lens and brain.

Despite seemingly consistent data from laboratory research, data from clinical studies is conflicting. Zetterberg described no association between cataract and ApoE gentoype after examining 502 cases of cataract and 187 controls.[199] Utheim et al. reported that cataract was associated with a lower prevalence of ApoE4 allele, though the examined sample was small (N=88) and highly selected.[200] Zubenko et al. found a significant reduction in the age-specific rate and lifetime cumulative incidence of age-related cataract in individuals >90 years old with preserved cognition, a group exhibiting successful aging.[136] Comparisons between our study and these previous studies is complicated by differences in the study population (e.g., mean age, age range, and source of participants from clinics vs. the general population), data source (e.g., medical records, self report, grading of cataract severity using standard photographs, direct measurement of lens transparency using a technique such as Scheimpflug photography), and statistical power. Moreover, because diagnosed cataract and cataract surgery are disease phenotypes which may have different associations with markers of aging and age-related chronic disease,[201] it is difficult to extrapolate results from studies of cataract to studies of lens transparency.

With the growing burden of dementia, consistency of results from laboratory studies, and inconsistency of results from previous population-based studies, it seems prudent to study lens transparency further as a noninvasive marker of brain aging. Beginning a new prospective cohort study correlating the lens to the brain would be highly desirable. This study should recruit initially cognitively intact older adults who have not undergone cataract surgery. If a participant were to undergo cataract surgery during follow-up, lens transparency could be determined

immediately before surgery and the explanted lens would be saved for histologic analysis. Several hypotheses could be tested using cross-sectional and prospective data collection. First, one could use positron emission tomography to determine if lens transparency was associated with amyloid deposition (using Pittsburgh-Compound B) or glucose uptake (using FDG-PET, a marker of brain metabolism). Second, one could study the lens in association with anatomic and physical findings drawn from magnetic resonance imaging and computed tomography, such as macrostructural changes (size of the ventricles; volume of grey matter, white matter, and cerebral spinal fluid; white matter grade) and microstructural integrity of the white matter (with diffusion tensor imaging). Third, because there is a wide range of cognitive performance at similar levels of neuropathology, it would be advantageous to test if lens transparency is strongly correlated with global cognition (e.g., using the Mini Mental Status Exam,) and psychomotor speed and visuomotor processing (e.g., using the Digit Symbol Substitution Test). This would provide data on the association of the lens to functional ability, which is clinically relevant and distinct from pathology. Fourth, lens transparency and markers of brain aging could be studied in association with systemic markers of glycation (e.g., advanced glycation end products), oxidation (e.g., isoprostanes), and cellular senescence (e.g., telomere length, p16) measured in blood. If an association between the lens and the brain were attenuated by these systemic markers it may suggest a common underlying mechanism for declining transparency and brain aging. Prospective data collection would allow one to correlate changes in all of these measures, and possibly assess whether cross-sectional lens transparency or changes in lens transparency predict death. If lens tissue was analyzed after cataract surgery, one could correlate lens molecules (e.g., amyloid deposition, crystalline integrity) with markers of brain aging. A study such as this may provide strong evidence for the lens being a non-invasive marker of brain aging.

Our findings also indicate that the lens may not be a strong indicator of vascular aging and disease, particularly arteriosclerosis and atherosclerosis. This is the first report of the association of lens transparency to CAC, aortic calcium, and carotid intima-media thickness. In population-based cohort studies, age-related cataract has been associated with diagnosed cardiovascular disease, diabetes, obesity, inflammation, smoking, sunlight exposure, and mortality.[135] Statin use has also been associated with a lower incidence of nuclear cataract, though it is unknown if this association is causal or correlative.[188] Nearly half of the participants in the HWS used lipid-altering drugs at the fourth EBT examination when lens transparency was assessed. If risk factors for atherosclerosis or cardiovascular disease are associated with lower transparency, and statin use is associated with greater transparency, it may be difficult to detect these associations in diseased individuals who use statins because the biological effects may obscure each other. Furthermore, it is unknown if associations found between cataract and disease markers would be similar in magnitude to possible associations between lens transparency and disease markers. Nonetheless, the consistency of results here implies that the lens may be an insensitive indicator of vascular aging.

Our data's validity is supported by our detecting as association between cataract surgery or lower lens transparency and diabetes, which is established in the ophthalmic epidemiology literature.[135] The mechanisms behind this association lend credence to the lens reflecting systemic aging and/or disease. With excess glucose in the body, formation of advanced glycation end products (AGEs), where glucose is non-enzymatically bound to proteins and lipids, occurs more often throughout an organism.[202-204] AGEs spur oxidation through free radical formation and inflammation through signaling by the receptor for advanced glycation end products.[205] In particular, these processes damage delicate tissues, such as the glomerulus, peripheral nerves,

and retina, leading to the diabetes-associated conditions of nephropathy, neuropathy, and retinopathy.[204] AGEs have also been associated with atherosclerosis and vascular stiffening by affecting collagen in vessel walls.[202,204] Epidemiologic studies illustrate that higher levels of serum carboxymethyl-lysine, a dominant AGE in serum and tissues, is associated with greater mortality, slow walking speed, anemia, and increased aortic pulse wave velocity in older adults.[206-209] The hallmark of AGE formation is a change in molecular structure with addition of glucose. Because crystallins maintain protein conformation and can themselves be altered by glycation, the lens may reflect systemic AGE production, and thus tissue integrity, throughout the body. Future studies should assess if lens transparency is associated with serum AGEs, such as carboxymethyl-lysine. This could be accomplished easily by adding lens measurements to epidemiologic studies in which AGEs have already been measured, such as the InCHIANTI study and the Baltimore Longitudinal Study of Aging.

An alternative explanation for our results is that we failed to detect true associations between the lens and markers of aging and disease due to inadequate power, bias, residual confounding, or misclassification. The age range of the HWS was narrow, possibly limiting inherent variability in lens transparency. This may have been exacerbated by the fact that cataract surgery has become relatively common due to efficiency, effectiveness, and high insurance coverage. Subsequently, participants may have been "removed" from the sample by treatment, which limits variability in lens transparency and decreases the number of participants eligible for direct lens assessment, further reducing power. Participants were also healthy survivors and may exemplify the "worried well" who participate in research studies and have favorable health behaviors. This may have limited variability in lens transparency and outcomes and/or introduced bias in an unknown direction. Because of the sample size and *a priori* desire to

conduct an exploratory analysis, we also chose to examine associations using tests of distribution rather than regression, so residual confounding may exist. Only women were included in this analysis. Although there is no suggestion in the literature that any potential associations examined here would differ by gender, it is unknown if gender is a modifier. Finally, it is possible that we misclassified lens transparency or outcomes, though this is unlikely. The participants' high cognitive function suggests they would not forget to report undergoing cataract surgery, and Scheimpflug photography provides a quantitative, unbiased, reproducible assessment of lens transparency. It also seems appropriate to categorize participants who underwent cataract surgery in the control group of lens transparency <90% or <95%. Finally, although 94% of the analytic sample was Caucasian, it is possible that admixture due to ancestry confounded the association with ApoE genotype.

This study does have several strengths. Although the narrow age range of the HWS may have limited variability in lens transparency and outcomes, it also limits variability in potential age-associated confounders. The rich historical data allowed us to conduct a retrospective longitudinal analysis to help establish temporality. The HWS employs validated protocols and highly experienced staff to gather data, and procedures have remained consistent throughout the study. Combined with the staff's long-time familiarity with study participants, this provides confidence that data collection was precise and accurate.

In conclusion, this is the first study to examine the association between directly-measured lens transparency and markers of atherosclerosis (CAC and carotid initima-media thickness) and markers of brain health, such as ApoE genotype and cognition. We found that lower lens transparency was associated with diabetes and potentially with ApoE4 genotype. Because lens transparency can be measured easily, cheaply, and non-hazardously, it has great potential for use

in examining human health, potentially across the lifespan. Future studies would benefit from serially measuring lens transparency and determining its association with established markers of aging and disease and emerging biomarkers of aging, such as telomere length,[201] AGEs, and insulin-like growth factor.

6.6 ACKNOWLEDGEMENTS

Authors' contributions: conception and design (JLS, AN, YPC, LHK, ABN); acquisition of data (JLS, AN, LHK, ABN); analysis and interpretation of data (JLS, AN, YPC, RMB, LPN, LHK, ABN); drafting of the manuscript (JLS, ABN); critical revision of the manuscript (JLS, AN, YPC, RMB, LPN, LHK, ABN); statistical analysis (JLS, RMB); obtaining funding (JLS, AN, YPC, LHK, ABN); administrative, technical, or materials support (AN, LHK, ABN); supervision (AN, LHK, ABN).

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6.7 TABLES

Table 6.7.1: Ocular characteristics of the Healthy Women Study Aging Lens Sub-study population

	Total N	N (%)	Mean (SD)	Median (IQR)	Minimum	Maximum
Bilateral cataract surgery	231	64 (27.7)				
Glaucoma	231	22 (9.5)				
Other eye disease	231	8 (3.4)				
Right eyes						
Nuclear cataract (%)	68		0.5 (1.1)	0.0 (0.4)	0.0	5.6
Cortical cataract (%)	68		7.5 (10.1)	3.7 (8.1)	0.2	50.2
Total transparency (%)	68		91.9 (10.6)	95.4 (8.0)	47.3	99.8
Contrast: No. letters read*	69		29 (5)	30 (3)	6.0	36.0
Acuity worse than 20/20[†]	56	47 (83.9)				
Left eyes						
Nuclear cataract (%)	72		0.6 (2.1)	0.1 (0.4)	0.0	15.5
Cortical cataract (%)	72		11.4 (14.5)	5.5 (12.3)	0.2	67.1
Total transparency (%)	72		88.0 (15.3)	94.1 (12.7)	32.2	99.8
Contrast: No. letters read*	69		28 (4)	29 (4)	18.0	36.0
Acuity worse than 20/20[†]	56	49 (87.5)				
Depth Perception[‡]						
Failed thick plate	69	3 (4.4)				
Failed medium plate	65	4 (6.2)				
Failed thin plate	61	21 (34.4)				

*Pelli-Robson test

[†]Bailey-Lovie distance visual acuity test

[‡]Frisby test

Table 6.7.2: Association of lens transparency at the 4th EBT examination to risk factors for coronary artery calcium and medications at 4th EBT examination

	Cataract surgery (N=231)			Cataract surgery and transparency (N=136)			Cataract surgery and transparency (N=136)		
Risk factor	**No (N=167)**	**Yes (N=64)**	**P**	**≥90% Transparent (N=39)**	**<90% Transparent (N=97)***	**P**	**≥95% Transparent (N=26)**	**<95% Transparent (N=110)***	**P**
Age, years, M (SD)	73.1 (1.7)	73.6 (1.6)	**0.05**	73.1 (1.8)	73.6 (1.6)	0.12	73.0 (1.5)	73.5 (1.7)	0.15
Smoking, N (%)	4 (2.6)	2 (3.6)	0.69	0 (0)	2 (2.3)	0.34	0 (0)	2 (2.0)	0.47
Waist circumference, cm, M (SD)	88.1 (13.1)	94.2 (15.7)	**<0.01**	89.3 (14.2)	91.3 (15.3)	0.49	88.3 (12.1)	91.3 (15.5)	0.38
Total cholesterol, mg/dL, M (SD)	221 (42)	213 (48)	0.29	211 (43)	220 (46)	0.29	213 (46)	219 (45)	0.58
HDL, mg/dL, M (SD)	68 (15)	68 (18)	0.78	68 (16)	69 (17)	0.73	68 (16)	69 (17)	0.65
LDL, mg/dL, M (SD)	129 (38)	122 (42)	0.27	120 (37)	128 (41)	0.33	122 (36)	126 (41)	0.65
Triglycerides, mg/dL, M (SD)	118 (49)	119 (54)	0.87	114 (50)	117 (54)	0.79	116 (50)	116 (54)	0.97
Glucose, mg/dL, M (SD)	103 (14)	105 (16)	0.42	104 (13)	105 (17)	0.73	102 (10)	105 (17)	0.23
Insulin, uIU/mL, M (SD)	13.3 (5.4)	14.9 (11.0)	0.32	13.5 (5.9)	13.6 (9.2)	0.92	12.9 (5.3)	13.8 (8.9)	0.55
Systolic BP, mmHg, M (SD)	125 (18)	123 (24)	0.63	121 (14)	123 (22)	0.52	121 (12)	123 (21)	0.58
Diastolic BP, mmHg, M (SD)	66 (9)	67 (13)	0.83	66 (9)	66 (11)	0.99	68 (9)	66 (11)	0.38
Pulse pressure, mmHg, M (SD)	58 (15)	56 (17)	0.47	55 (12)	57 (16)	0.48	53 (12)	57 (15)	0.24
Lipid medication, N (%)	73 (46.5)	25 (43.9)	0.73	22 (56.4)	34 (38.2)	0.06	14 (53.9)	42 (41.2)	0.25
Blood pressure medication, N (%)	76 (48.4)	25 (43.9)	0.56	22 (56.4)	38 (42.7)	0.15	15 (57.7)	45 (44.1)	0.22
Diabetes medication, N (%)	7 (4.5)	9 (15.8)	**<0.01**	1 (2.6)	12 (13.5)	0.06	0 (0)	13 (12.8)	**0.05**
Diabetes by meds/glucose, N (%)	12 (7.2)	11 (17.2)	**0.02**	2 (5.1)	14 (14.4)	0.13	0 (0)	16 (14.6)	**0.04**
Interleukin-6, median (IQR)	1.89 (1.21)	2.16 (1.84)	0.28	2.01 (1.75)	2.16 (1.63)	0.61	1.97 (1.74)	2.15 (1.73)	0.41

*Includes participants who underwent cataract surgery. M (SD) = mean (standard deviation).

Table 6.7.3: Association of lens transparency at 4th EBT examination to risk factors for coronary artery calcium and medications at baseline HWS examination

	Cataract surgery (N=231)			Cataract surgery and transparency (N=136)			Cataract surgery and transparency (N=136)		
Risk factor	**No (N=167)**	**Yes (N=64)**	**P**	**≥90% Transparent (N=39)**	**<90% Transparent (N=97)***	**P**	**≥95% Transparent (N=26)**	**<95% Transparent (N=110)***	**P**
Age, years, M (SD)	47.5 (1.6)	47.9 (1.5)	0.07	47.4 (1.6)	47.9 (1.6)	0.10	47.2 (1.3)	47.9 (1.6)	0.06
Smoking, N (%)	35 (21.1)	21 (32.8)	0.06	10 (25.6)	26 (26.8)	0.89	8 (30.8)	28 (25.5)	0.58
Body mass index, kg/m^2, M (SD)	23.2 (4.2)	24.5 (4.7)	0.05	23.8 (4.7)	24.2 (4.4)	0.64	23.3 (2.8)	24.2 (4.8)	0.33
Total cholesterol, mg/dL, M (SD)	182 (33)	186 (37)	0.40	185 (27)	185 (35)	0.95	186 (28)	184 (34)	0.82
HDL, mg/dL, M (SD)	60 (14)	58 (14)	0.21	63 (15)	58 (13)	0.09	61 (15)	59 (14)	0.50
LDL, mg/dL, M (SD)	107 (28)	110 (32)	0.51	107 (29)	110 (31)	0.66	110 (30)	109 (30)	0.82
Triglycerides, mg/dL, M (SD)	74 (34)	90 (58)	**0.03**	72 (25)	84 (53)	0.07	73 (24)	83 (51)	0.15
Glucose, mg/dL, M (SD)	85 (11)	91 (39)	0.24	85 (10)	90 (32)	0.21	85 (10)	89 (30)	0.21
Insulin, uIU/mL, M (SD)	7.3 (4.2)	8.3 (6.6)	0.29	7.0 (3.8)	8.0 (5.7)	0.21	6.2 (2.7)	8.1 (5.6)	**0.02**
Systolic BP, mmHg, M (SD)	106 (10)	110 (14)	0.09	108 (11)	108 (13)	0.94	107 (11)	108 (12)	0.58
Diastolic BP, mmHg, M (SD)	71 (8)	71 (8)	0.66	71 (8)	71 (8)	0.78	70 (9)	71 (8)	0.45
Pulse pressure, mmHg, M (SD)	35 (7)	38 (10)	**0.05**	37 (8)	37 (9)	0.88	37 (6)	37 (9)	0.91

*Includes participants who underwent cataract surgery. M (SD) = mean (standard deviation).

Table 6.7.4: Association of lens transparency at 4th EBT examination to total coronary artery calcium score at 1st, 2nd, 3rd, and 4th EBT examinations

Total coronary artery calcium score	Cataract surgery (N=231)			Cataract surgery and transparency (N=136)			Cataract surgery and transparency (N=136)		
	No (N=167)	Yes (N=64)	P	≥90% Transparent (N=39)	<90% Transparent (N=97)*	P	≥95% Transparent (N=26)	<95% Transparent (N=110)*	P
4th EBT (N=193)									
0 (N=56), N (%)	44 (30.8)	12 (24.0)	0.60	10 (30.3)	17 (21.8)	0.49	4 (19.1)	23 (25.6)	0.79
1-99 (N=69), N (%)	51 (35.7)	18 (36.0)		13 (39.4)	29 (37.2)		8 (38.1)	34 (37.8)	
100+ (N=68), N (%)	48 (33.6)	20 (40.0)		10 (30.3)	32 (41.0)		9 (42.9)	33 (36.7)	
Score, median (IQR)	32.1 (196.7)	54.2 (225.2)	0.75	30.3 (164.8)	59.0 (231.7)	0.74	53.1 (198.5)	40.2 (226.3)	0.62
3rd EBT (N=225)									
0 (N=85), N (%)	64 (39.5)	21 (33.3)	0.48	15 (38.5)	31 (32.6)	0.64	8 (30.8)	38 (35.2)	0.27
1-99 (N=86), N (%)	58 (35.8)	28 (44.4)		13 (33.3)	40 (42.1)		8 (30.8)	45 (41.7)	
100+ (N=54), N (%)	40 (24.7)	14 (22.2)		11 (28.2)	24 (25.3)		10 (38.5)	25 (23.2)	
Score, median (IQR)	6.8 (99.9)	10.3 (92.4)	0.63	6.9 (105.1)	10.3 (105.7)	0.85	21.0 (149.7)	9.1 (92.6)	0.66
2nd EBT (N=200)									
0 (N=93), N (%)	73 (49.3)	20 (38.5)	0.39	18 (47.4)	33 (40.7)	0.73	11 (44.0)	40 (42.6)	0.30
1-99 (N=79), N (%)	55 (37.2)	24 (46.2)		14 (36.8)	36 (44.4)		8 (32.0)	42 (44.7)	
100+ (N=28), N (%)	20 (13.5)	8 (15.4)		6 (15.8)	12 (14.8)		6 (24.0)	12 (12.8)	
Score, median (IQR)	1.0 (24.6)	2.8 (55.2)	0.33	2.4 (50.1)	2.8 (41.5)	0.95	5.2 (63.9)	1.9 (28.5)	0.47
1st EBT (N=229)									
0 (N=128), N (%)	98 (59.4)	30 (46.9)	0.20	24 (63.2)	48 (49.5)	0.27	13 (52.0)	59 (53.6)	0.43
1-99 (N=74), N (%)	48 (29.1)	26 (40.6)		9 (23.7)	37 (38.1)		7 (28.0)	39 (35.5)	
100+ (N=27), N (%)	19 (11.5)	8 (3.5)		5 (13.2)	12 (12.4)		5 (20.0)	12 (10.9)	
Score, median (IQR)	0 (13.0)	1.5 (37.9)	0.09	0 (29.0)	1.0 (30.9)	0.15	0 (30.1)	0 (18.0)	0.88

*Includes participants who underwent cataract surgery.

Table 6.7.5: Association of lens transparency at 4th EBT examination to ApoE genotype and Modified Mini Mental Status Exam score

	Cataract surgery (N=231)			Cataract surgery and transparency (N=136)			Cataract surgery and transparency (N=136)		
	No (N=167)	Yes (N=64)	P	≥90% Transparent (N=39)	<90% Transparent (N=97)*	P	≥95% Transparent (N=26)	<95% Transparent (N=110)*	P
ApoE status, N (%)									
2,2	1 (0.6)	0 (0)	0.20	0 (0)	0 (0)	**0.01**	0 (0)	0 (0)	**0.04**
3,2	20 (12.2)	5 (7.9)		4 (10.5)	8 (8.3)		1 (3.9)	11 (10.2)	
3,3	111 (67.7)	41 (65.1)		28 (73.7)	62 (64.6)		22 (84.6)	68 (63.0)	
4,2	4 (2.4)	0 (0)		2 (5.3)	0 (0)		0 (0)	2 (1.9)	
4,3	25 (15.2)	17 (27.0)		3 (7.9)	26 (27.1)		2 (7.7)	27 (25.0)	
4,4	3 (1.8)	0 (0)		1 (2.6)	0 (0)		1 (3.9)	0 (0)	
2,2 or 3,2 or 3,3	132 (80.5)	46 (73.0)	0.22	32 (84.2)	70 (72.9)	0.17	23 (88.5)	79 (73.2)	0.10
4,2 or 4,3 or 4,4	32 (19.5)	17 (27.0)		6 (15.8)	26 (27.1)		3 (11.5)	29 (26.9)	
3MS score, median (IQR)	97 (4)	96 (6)	**0.02**	98 (5)	97 (5)	0.09	98 (5)	97 (5)	0.33

*Includes participants who underwent cataract surgery.

7.0 DISCUSSION

7.1 SUMMARY, CONCLUSIONS, AND FUTURE RESEARCH

The main goal of these studies was to clarify the role of LTL and lens transparency as biomarkers in population studies of human aging. The results provide new evidence on the overall value of these biomarkers in aging research, particularly their specificity reflecting aspects of human aging.

7.1.1 Leukocyte telomere length

LTL is proposed as a biomarker of aging because it ostensibly records inflammation and oxidative stress,[23,25,158] but the evidence for LTL as a general biomarker of aging remains unclear. Notably, previous studies have almost exclusively attempted to associate LTL with clinically diagnosed disease in individual organ systems, usually categorized as present or absent, despite the fact that chronic disease can build throughout the body over time. Subsequently, we chose to study the association of LTL to a marker of disease burden, the physiologic index of comorbidity, which tabulates the presence and severity of common chronic age-related disease across five major physiologic systems. An important aspect of the five systems and their measurement is that they both capture changes from age and age-related

chronic disease; they tend to change with age even without other external risk factors. To the extent that an index across systems might capture an underlying propensity to age-related changes in all systems, a marker of fundamental aging processes such as LTL should be associated with it. Using this index we demonstrated that LTL was strongly associated with disease burden independent of diagnosed chronic conditions. LTL was less strongly associated with disease in individual systems, except possibly carotid intima-media thickness, and was not associated with diagnosed chronic conditions or a count of diagnosed chronic conditions. In this study LTL was associated with disease burden after removing carotid thickness from the physiologic index of comorbidity. This sensitivity analysis was conducted because vascular disease appeared to drive the association of the index with LTL, and we wished to see if LTL was associated with disease burden independent of vascular disease. The results imply that LTL might indicate widespread incremental changes in organ structure and function from age and age-related chronic disease in older adults independent of vascular disease.

There are a large number of cross-sectional studies on LTL that demonstrate few consistent results. Given this new data, it is appropriate to ask, are more studies of LTL warranted, and, if so, how should these studies be designed? This data implies that there is more to learn about LTL's potential contribution to human biology if it is studied in association with less conventional measurements like disease burden rather than standard measurements such as clinically diagnosed disease. Telomere length is a fundamental regulator of cellular senescence in laboratory studies yet it remains unknown if it contributes to human longevity, apart from its involvement in cancer. It would be surprising if telomere length was not even a small determinant of human longevity given the strength and consistency of results from the laboratory. More research in human populations is needed to test this important hypothesis.

Moreover, because LTL can be sampled easily, cheaply, and throughout the lifespan, characteristics which are advantageous for translation of an aging biomarker, it seems worthwhile to continue studying LTL in epidemiology.

The most valuable data on LTL would come from longitudinal studies of changes in LTL – these studies allow more accurate modeling of LTL dynamics and may illustrate age-associated biological mechanisms better than studies with a single measurement of LTL. It would be advantageous to determine predictors of change in LTL, which necessitates modeling change in LTL as an outcome. Strong data that LTL reflects biological aging would come from studies modeling simultaneous changes in LTL, systemic markers of inflammation and oxidation, and disease burden. One could model the association between changes in disease burden and systemic markers of inflammation and oxidation, such as interleukin-6 and isoprostanes, and add change in LTL to the model to see if this mediates the association. If changes in LTL and changes in systemic markers of oxidation/inflammation were modeled jointly as predictors of aging (e.g., mortality, frailty, disease burden, etc.), it would be possible to test for interactions between LTL and oxidation markers. This may identify joint contributions of declining LTL and inflammation or oxidation on influencing aging, which may provide evidence in humans that inflammation and oxidation may act through changes in LTL. These studies require follow-up longer than 10 years to circumvent the problem of measurement error confounding observed changes in LTL. At first, data may be derived from measuring LTL in previously gathered blood samples from existing cohort studies. Because of the large inter-individual variance in LTL and sensitivity of results to measurement error introduced by various assays, cohorts with the widest age range possible would be ideal for change studies. The sample should be highly phenotyped to allow adjustment for potential confounders, which is chronically absent from studies of LTL.

Large sample size will confer more power to counteract variance introduced by measurement method, and will also provide stability for models containing many potential covariates. Announcement of future measurement of LTL in nearly 100,000 members of the Kaiser Permanente health maintenance organization provides confidence that data of this magnitude may soon be available. In addition to the benefits listed above, this sample will likely provide data that is more generalizeable and can be linked to other useful datasets, such as the National Death Index, which would enable survival analysis. Unfortunately, because Kaiser data is derived from clinical sources, it is unlikely that the dataset will include measurement of subclinical disease, which we have shown is critical to detecting significant associations, or other characteristics traditionally assessed in a research setting, such as standardized functional measures (e.g., gait speed, grip strength). Using pooled data from research studies with LTL measured with similar methods, such as studies included in the CHARGE consortium and related European cohorts, may increase power while providing more detailed measurements including subclinical disease. This type of pooling was used for a recent genome-wide association study of LTL.[210] Pooling would enable researchers to construct a similar index of disease burden in a sample that also has adequate numbers of diagnosed chronic conditions to test, with adequate power, if LTL is associated with age-related chronic disease burden and/or diagnosed chronic conditions. Furthermore, using the richness of these datasets, it would be possible to study if LTL is associated with a tabulation of functional health, such as frailty, which has been related to disease burden.[211] In addition to studying the strength of the association of LTL with disease burden, frailty, and death, it would be worthwhile to determine the accuracy of LTL predicting these outcomes using the area under the receiver-operator curve, particularly if LTL adds

additional predictive ability to models including age and other easily acquired indicators of health status, such as BMI and smoking history.

Our analysis of sub-components of the index also suggests that in older adults LTL may be most strongly associated with processes which contribute to atherosclerosis and/or hypertension specifically, and perhaps processes which contribute to structural and physiological changes in other organs, such as accumulation of white matter hyperintensities. This is consistent with some previous cross-sectional studies which show atherosclerosis is associated with shorter LTL.[33,51,53,54,56,57,63,71-75] Further research on LTL and atherosclerosis, provided phenotypes were carefully considered, would likely support this association. Because so many studies on LTL and atherosclerosis have been conducted more studies of this association are a lower priority.

Additional research is necessary, though, to confirm if LTL reflects brain aging because the few previous studies of LTL and the brain have used very different study populations.[81-85] Furthermore, these previous studies have only used cognitive function or clinically-defined dementia as phenotypes rather than anatomic, physiologic, or biologic markers of brain aging. If LTL was associated with brain pathology it may stand as a particularly useful marker to explore relationships between the brain, immune system, and aging, which are sorely needed given the lack of defined risk factors for brain aging. It would be advantageous to study the association of LTL to an *in vivo* marker of brain aging, such as amyloid plaques quantified with Pittsburgh-Compound B or glucose uptake using FDG;[212] macrostructural changes in the size of ventricles, grey and white matter volume, CSF volume, and white matter hyperintensity; and microstructural integrity of the white matter using diffusion tensor imaging. Some of these associations may be studied if LTL was measured in the Ginko Evaluation of Memory (GEM) Study, which included anatomic imaging and measurement of amyloid using Pittsburgh-

Compound B in several hundred participants, and could identify a particular niche where LTL might be a useful biomarker.

From a methodological perspective, these results illustrate that it is necessary to study a biomarker against many outcomes to determine its validity. Here, we employed an index that combined several other measures using a particular scoring method, continuous measurements of disease in individual organ systems, dichotomous classification of diagnosed diseases, and a count of diagnosed diseases. This list of outcomes is not comprehensive, and there are many methods to combine measurements into an aggregate score, but we nonetheless demonstrated different associations between LTL and all of these outcomes. These different associations allowed us to clarify which aspects of age-related disease LTL may accurately reflect, which would not have been possible if a single outcome definition were used.

How useful is LTL as a biomarker of aging when evaluated using the four criteria (statistical, biological, clinical, and experimental) proposed in **2.4** and **2.5**? Although it may add additional information in a predictive model, LTL is likely a weaker predictor of incident events than chronologic age. Subsequently, LTL may not be an advantageous biomarker to incorporate into predictive models because it poorly fulfills the statistical criterion. LTL can be measured relatively cheaply, easily, and repeatedly, so it fulfills the clinical criterion, but because LTL varies widely between species, it is questionable whether it fulfills the experimental criterion.

LTL may be most advantageous to human population studies when considered using the biological criterion. Basic science studies clearly indicate that telomere shortening is a fundamental regulator of cellular senescence and an important safeguard against tumorigenesis. In human population studies, the relevance of LTL remains equivocal. In epidemiology, LTL is typically measured at only one point in time. It is also often used as a surrogate for telomere

length in other tissues, and although there is evidence that telomere lengths are synchronized throughout the body, it remains possible that LTL does not reflect telomere length in every tissue, particularly because its measurement usually displays average telomere length and obscures the distribution of telomere lengths in a population of cells. It is possible that telomere length sampled from tissues more proximal to the physiologic systems in the index would be more strongly associated with disease in those systems.

Human population studies of LTL remain in their infancy. Subsequently, much can be learned about the biology of human telomere dynamics from future research. Salient questions to answer include: What is the normal rate of telomere shortening in a human population? What is the variance in telomere shortening? What risk factors accelerate telomere shortening and what protective factors ameliorate shortening? Is the rate of telomere shortening predictive of specific age-associated outcomes or overall organismal aging measured using different constructs? If there truly is a dichotomy between aging and cancer, is telomere shortening the central regulator of this dichotomy? Answering these questions requires longitudinal measurement of telomere length, likely over decades, in well-characterized populations. This could position telomere length as a prime candidate for exploration using the growing subfield of lifecourse epidemiology. It would also behoove researchers to standardize measurement techniques while developing assays with higher throughput and higher accuracy to facilitate comparisons across studies and pooling for meta-analysis. If possible, telomere length should also be sampled from non-hematopoetic tissues to learn whether tissue source impacts the strength of associations. With these advancements, researchers will be able to more confidently assess telomere length as a biomarker of aging in humans.

7.1.2 Lens transparency

The lens is a fascinating tissue. Present from before birth until death with little turnover, it is also one of the few tissues that directly interacts with the internal and external environments. It is nearly homogenous and comprised almost entirely of a few types of proteins, crystallins, which also exist throughout the body. As heat shock proteins, crystallins critically maintain the conformation and function of themselves and other proteins, and play a part in an organism's successful response to stress throughout the lifespan. Because it is optically clear, the lens can be measured easily, cheaply, and non-invasively using accurate and reproducible imaging techniques. Importantly, lens transparency can be measured continuously using imaging to distinguish a perceived aging phenotype, declining transparency, from a disease phenotype, diagnosed presence of cataract or cataract surgery. These characteristics provide both a biological and practical basis for using lens transparency as a biomarker of aging in population studies of human aging.

Despite its theoretical and actual advantages, the lens has not been validated as a biomarker of aging. We studied the lens in association with markers of aging and age-related disease to advance it as a putative biomarker of aging. We used data from the Health, Aging, and Body Composition Study and Age-Related Maculopathy Ancillary study to differentiate between diagnosed cataract and cataract surgery, disease phenotypes, and AREDS-graded lens transparency, an aging phenotype, while determining the association of these lens measures to LTL. The studies also provided a wealth of phenotypic and biologic data to consider as potential confounders. We found that participants with highly transparent lenses, a rare group comprising less than 3% of the sample, had much longer LTL than participants with lower lens transparency, but that cataract surgery was not associated with LTL. It was also remarkable that the difference

in mean LTL between participants with highly transparent and less transparent lenses was nearly 1,000 bp, a much greater difference in mean LTL than seen in the literature with stratification by other variables. Participants with highly transparent lenses also had no history of smoking or clinically-diagnosed cerebrovascular disease or cardiovascular disease despite being over 70 years old. This result implies that high lens transparency may be a rare longevity phenotype useful for identifying individuals who have aged exceptionally well in other tissues. The results also suggest that specific measurements of lens transparency may register aging more accurately than dichotomous classification using presence or absence of cataract. As the first study of the association of the lens with LTL it is difficult to set these results in context, but they lend credence to exploring the lens as a biomarker of aging.

In the Healthy Women Study, we examined the association of the lens with a more extensive battery of markers of aging and disease to provide broader evidence for the lens as a biomarker of aging. The HWS afforded measurement of CAC using EBT, a strong predictor of cardiovascular disease and death. It also provided EBT-measurement of aortic calcium, which also increases with age but is less associated with cardiovascular disease risk factors, and carotid intima-media thickness, which increases with age, high blood pressure, and atherosclerosis. Because the study is ongoing we were able to measure lens transparency with high accuracy using Scheimpflug photography when participants returned for their fourth EBT examination. As a longitudinal cohort study, the HWS also enabled us to conduct both cross-sectional and retrospective longitudinal analyses.

In the HWS, we found that neither cataract surgery nor lens transparency was associated with most examined risk factors for coronary atherosclerosis or CAC, aortic calcium, or carotid intima-media thickness, measured concurrently or previously. There was a relatively consistent

association between lower transparency and markers of diabetes, which was expected given the established relationship between diabetes and cataract. Finally, we observed that transparency was associated with ApoE genotype, and that greater transparency may be associated with a lower frequency of carrying an ApoE4 allele and better cognitive function.

Taken together, these results are promising. If the associations are true it suggests that lens transparency may be a viable biomarker of aging, particularly as an indicator of brain aging and diabetes, but not coronary atherosclerosis or related risk factors. Histological studies support a connection between the lens and brain aging, particularly Alzheimer's type pathology characterized by protein aggregation and deposition, due to the presence and similar activity of crystallins in the lens and brain. Two previous studies examining an association between cataract and ApoE genotype, both of which used a selected sample of participants from eye clinics, found no association between cataract and ApoE4 genotype or that cataract was associated with lower odds of ApoE4 genotype.

Given these previous studies and our own data, lens transparency warrants future investigation as a noninvasive marker of brain aging. Beginning a new prospective cohort study correlating the lens to the brain would be highly desirable. This study should recruit initially cognitively intact older adults who have not undergone cataract surgery. If a participant were to undergo cataract surgery during follow-up, lens transparency would be determined immediately before surgery and the explanted lens would be saved for histologic analysis. Several hypotheses could be tested using cross-sectional and prospective data collection. First, one could determine if lens transparency was associated with amyloid measured *in vivo* using Pittsburgh-Compound B or glucose uptake using FDG-PET. Second, one could study the lens in association with anatomic and physical findings drawn from MRI and CT, such as changes in the size of the

ventricles, grey and white matter volume, CSF volume, white matter grade, and microstructural integrity of the white matter (with diffusion tensor imaging). These are strong markers of macro and microstructural integrity and possibly cognitive reserve. Third, because there is a wide range of cognitive performance at similar levels of neuropathology, it would be advantageous to test if lens transparency is strongly correlated with global cognition (e.g., using the Mini Mental Status Exam,) and psychomotor speed and visuomotor processing (e.g., using the Digit Symbol Substitution Test). This would provide data on the association of the lens to functional ability, which is clinically relevant and distinct from pathology. Fourth, lens transparency and markers of brain aging could be studied in association with systemic markers of glycation (e.g., AGEs), oxidation (e.g., isoprostanes), and cellular senescence (e.g., telomere length, p16) measured in blood. If an association between the lens and the brain were attenuated by these systemic markers it may suggest a common underlying mechanism for declining transparency and brain aging. Prospective data collection would allow one to correlate changes in all of these measures, and possibly assess whether cross-sectional lens transparency or changes in lens transparency predict death. If lens tissue was analyzed after cataract surgery, one could correlate lens molecules (e.g., amyloid deposition, crystalline integrity) with markers of brain aging. A study such as this may provide strong evidence for the lens being a non-invasive marker of brain aging.

Would it be useful to conduct more studies on the association of the lens to cardiovascular disease? Population-based studies suggest that cataract is associated with cardiovascular disease, but CAC, aortic calcium, and carotid intima-media thickness have not been studied previously. It is possible that age-related cataract is associated with diagnosed cardiovascular disease but not subclinical markers of cardiovascular disease specifically. Confounding by statin use may have occurred. Statins have been associated with lower incidence

of nuclear cataract but not overall cataract: in 1,299 participants of the Beaver Dam Eye Study, five-year incidence of nuclear cataract was 12.2% in statin users compared with 17.2% in nonusers (OR 0.55, 95% CI 0.36-0.84), adjusted for age.[188] When only never-smokers without diabetes were assessed, the age-, sex-, and lipid-adjusted OR for nuclear cataract was 0.40 (95% CI 0.18-0.90). Because half of the HWS participants were using statins, and these women were predominantly non-smokers without diabetes, this may have counterbalanced a significant positive association between cataract and risk factors for atherosclerosis and resulted in non-detection of a significant association. Nonetheless, our results were relative consistent in that neither cataract surgery nor lens transparency were associated risk factors for coronary atherosclerosis or CAC, aortic calcium, or carotid intima-media thickness. Significant differences detected between groups were in general small and did not demonstrate a clear pattern of better transparency being associated with a favorable cardiovascular health profile (e.g., lower LDL, lower triglycerides, higher HDL, smaller waist circumference).

It would be helpful to achieve greater certainty of the specificity of lens transparency as a biomarker of aging, particular in regard to cardiovascular disease. Creating new cohort studies seems unwarranted, but using existing cohorts could be feasible. In particular, it would be advantageous to study the lens in association with additional cardiovascular phenotypes. Ankle brachial index is a suitable outcome and a marker of peripheral vascular disease, a different phenotype. Pulse wave velocity, an ultrasound-based marker of vascular stiffening more intimately tied to blood pressure, is strongly associated with age independent of disease and would be another worthy target for investigation. Ankle brachial index and pulse wave velocity have been measured in many large cohort studies of aging and cardiovascular disease.[213] If data was gathered on cataract in these studies, it could be used to conduct a retrospective longitudinal

study to determine if cataract is associated with facets of vascular aging and disease. If cataract data was not ascertained previously, participants could be re-contacted and diagnosis of cataract or cataract surgery could be ascertained via self-report. Alternatively, these studies and others could add measurements of lens transparency and test if current lens transparency reflects previous levels of vascular disease, or, if clinical examinations are ongoing, if prospectively gathered lens transparency is associated with concurrent changes in vascular markers.

An alternative explanation for the null results from the HWS is that we committed a type II error due to inadequate power, bias, residual confounding, or misclassification. The HWS had a narrow age range, which could have limited variability in lens transparency and other markers. This would decrease power to detect a significant association. The low end of the transparency spectrum may also have been eliminated because many participants had cataract surgery fairly early, which has become relatively ubiquitous. Although only women were included in this analysis, and there is no suggestion in the literature that associations would differ by gender, it is still possible that gender modifies any real associations. Subsequently, this bias, as well as other unknown biases due to sample selection, may have influenced results. It is possible that we misclassified lens transparency, though this is unlikely because participants should accurately remember if they underwent cataract surgery (particularly because their cognitive function was high when measured with the 3MS), and Scheimpflug photography provides an accurate measurement of lens transparency.

Is lens transparency a valid biomarker of aging in human population studies? To determine whether lens transparency adds meaningful data to statistical models predicting age-related outcomes, in addition to the new specific studies described above concerning brain and vascular aging, an efficient first approach may be analysis of data from studies on the

epidemiology of cataract. Although these studies often lack very sensitive measurements of lens transparency, such as Scheimpflug photography, they are generally large (for increased power), tabulate other phenotypic data (for examination as outcomes or for adjustment in models as confounders), and include younger participants (which provide a wider age span and potential for follow-up for lifecourse analysis). It may be possible to embed new measurements in these studies with re-enrollment to convert the studies into aging cohort studies. If resources were not available to enroll the entire cohort, participants could be selected for enrollment in case-cohort studies, which are more efficient but can maintain statistical robustness.

Practically, the lens has many advantages. It can be measured easily, reliably, and repeatedly using a variety of methods, including direct imaging with photography and grading against standard cataract photos. Subsequently, it should be relatively easy to incorporate lens measurements into existing human studies. Lower order mammals and some non-mammalian model systems, such as Zebra fish, have lenses which might serve to validate results from human studies or provide preliminary data for human studies. Indeed, imaging devices have been used successfully to provide quantitative lens data from mice. Furthermore, model systems can be used to conduct experiments, allowing researchers to determine if changes in genes or the environment may be concurrently reflected in the lens and other tissues, which may suggest that the lens and other tissues are linked. For example, caloric restriction has been shown to delay many age-associated changes as well as reduce the incidence of cataract. With increased incorporation of sensitive lens measurements into animal and human studies, researchers will be able to validate the lens as a biomarker of aging, and potentially position it for use as a tool in studies hoping to optimize aging.

7.1.3 Studying aging using the extremes versus the mean

Epidemiology is concerned with studying health patterns in populations. Typically, this is achieved by measuring the distribution of risk factors and outcomes in general population cohorts and noting associations between risk factor variation and outcome variation. Essentially, it is a study of the association between the mean level of risk factors and the mean level of outcomes: when one mean increases the other mean increases, and when one mean falls the other mean falls. A benefit of studying general populations is that data are more generalizable. A drawback to studying general populations is that within a distribution there can exist a great deal of heterogeneity. Although heterogeneity provides variance, and differences cannot be detected without variance, it also introduces a higher amount of background noise which can obscure detecting associations of interest.

Rather than studying differences in means from wide distributions, another way to detect differences is by studying means from less variable but extremely divergent distributions. More simply put, rather than compare people within a large general population, instead focus on the outliers, those that are highly different from the average. Although data derived from outliers may not be as generalizable, one often finds interesting associations at the extremes because there is a large difference between the mean of the general population and the mean of the outliers. Furthermore, gathering a small number of people at an extreme may create a more homogeneous phenotype, dampening the noise effect which can obscure associations at the cost of reducing statistical power.

These differences in approach, studying outliers vs. studying the general population mean, have particular relevance to aging research. As discussed in the Background of this dissertation, the crux of aging research is defining what is aging vs. what is disease. As opposed

to basic research in model systems where distinct phenotypes can literally be engineered or clinical research where subjects are recruited due to their expression of overt disease, it is difficult in population-based aging research to classify who exactly we are studying. Is it the normal aged? The diseased aged? The long lived who are healthy? The long lived who are sick? Typically, the consequence of this identity crisis is that we doggedly try to tease apart groups in a general population or tease apart an aging phenotype from a disease phenotype by relying on physical tools (more precise measurements, measurements at finer levels) or statistical tricks (increasing sample size to afford more variability and increased power). The other solution is to intelligently design studies which force distributions as far apart as possible by focusing on the extremes: people who age very rapidly and people who age very slowly. Advancement of LTL and lens transparency as biomarkers of aging in epidemiology may be best achieved by examining the extremes.

At one end of the aging spectrum are people who age rapidly, often lumped into a group of progeroid syndromes. These syndromes, such as Werner's syndrome, Hutchinson-Gilford Progeria Syndrome (HGPS), and Xeroderma pigmentosum (XP), have characteristics which appear like accelerated aging. They also allow probing of distinct molecular pathways due to their genetic origins. For example, in Werner's syndrome, which is caused by a mutation in the *WRN* gene, patients have a faulty 5'→ 3' DNA helicase and exonuclease. Interestingly, the molecular hallmark of Werner's syndrome is accelerated telomere attrition, and one clinical hallmark is that nearly 100% of patients suffer from early onset of bilateral cataracts.[40,180] HGPS is characterized by a defect in lamin A (coded by *LMNA)*, a cytoskeletal protein which supports the nuclear envelope.[214] The defect causes accumulation of the protein around the nuclear envelope, misshaping of the envelope, improper interaction between the envelope and adjacent

chromatin, disordered nuclear function, and inability of the cell to divide. HGPS patients have short stature, thinned skin, low frequency conductive hearing loss, growth hormone deficiency, insulin resistance, and many signs of cardiovascular aging including age-associated elevated blood pressure, reduced vascular compliance, decreased ankle–brachial indexes, and adventitial thickening, but usually normal cognition.[215] Patients die at an average age of 13 years old from myocardial infarction or stroke due to massively accelerated arteriosclerosis. XP can manifest from one of many point mutations in genes encoding proteins involved in nucleotide excision repair.[216,217] The genomic instability causes apoptosis and mutagenesis in many tissue types and clinically manifests as neurodegeneration (loss of neurons), endocrine dysfunction (loss of somatotrophic axis), photoaging of the skin and eyes, proliferation of cutaneous tumors, and hematopoetic failure (replicative senescence of hematopoetic stem cells). The drawback of using these syndromes to probe aging is that there are small numbers of patients (limiting power) and aspects of their presentation are different from "normal" aging. The syndromes can be replicated, though, in model systems, particularly rodents, which may afford studying certain aspects of their biology in association with LTL and lens transparency.

At the other end of the aging spectrum are people who age slowly. These people have been described as exhibiting longevity, healthy aging, or exceptional survival, among other terms which are not necessarily interchangeable. Opportunities to study the epidemiology of healthy aging are increasing rapidly.[218] The Long Life Family Study recruited nearly 5,000 individuals clustered in families with exceptional longevity (and their spouses) to participate in a study of the genetics of healthy aging. Compared to age-matched controls from population-based cohorts not selected for longevity, Long Life Family Study participants exhibit better health.[219] LTL has been measured in most Long Life Family Study participants and analysis of LTL heritability,

genetic associations, and associations with other phenotypes is underway. Centenarians have been gathered in the New England Centenarian Study. By virtue of living to be at least 97 years old, these adults exhibit exceptional longevity. LTL was examined in 36 members of this study, specifically comparing 19 "healthy" centenarians with physical function in the independent range and the absence of hypertension, congestive heart failure, myocardial infarction, peripheral vascular disease, dementia, cancer, stroke, chronic obstructive pulmonary disease, and diabetes to 19 "unhealthy" centenarians with physical function limitations and ≥ 2 of the above conditions. Healthy centenarians had significantly longer telomeres than did unhealthy centenarians ($p=0.0475$).[178] In another study of 32 supercentenarians (age 110-119), it was noted that 88% had cataracts.[220] What is surprising here is that 12% did not have cataracts despite being 110-119 years old. Seventh-Day Adventists, a self-selected cohort who adhere to strict dietary, social, and religious customs, and the Okinawan Japanese, a geographically and ethnically defined cohort also with a unique dietary, social, and religious makeup, have been found to exhibit exceptional longevity compared to general population references.[221-223] LTL and lens transparency have not been examined in these groups. Other populations of interest include long lived individuals derived from general population cohorts, such as members of the Cardiovascular Health Study-All Stars study. The All Stars study re-recruited 1,677 participants of the Cardiovascular Health Study who were alive in 2005-2006 to assess cognitive and physical function.[224] With a mean age of 85 years old, an age range of 77-102 years old, and nearly two decades of extensive phenotyping, the All Stars could be a particular interesting population within which to examine LTL and lens transparency. Another approach is pooling general population cohorts to achieve a sufficiently large sample of long lived individuals, as

was done in the CHARGE consortium.[225] Clearly there are many opportunities to explore LTL and lens transparency among these extremes.

In the future, epidemiologists who study aging will employ a number of tools to more sensitively probe human biology. At the epicenter of the toolbox will be classical epidemiologic techniques employed in artful ways, like studying extremes of the population. Second, better assays and imaging systems will enable researchers to more accurately and specifically quantify biomarkers of interest. High-throughput systems will allow epidemiologists to link large populations with deep phenotyping to variations in molecules and genes. Finally, advancing statistical techniques, such as joint modeling, longitudinal trajectory latent class analysis, mixed modeling, and lifecourse analysis will shift our rudimentary representation of biology toward a more sophisticated representation. With these new tools epidemiologists will help uncover the secrets to living a healthy, long life and be integral to the design, implementation, and assessment of interventions to promote longevity.

7.2 PUBLIC HEALTH IMPLICATIONS

The world's population is aging at an increasing rate. This demographic transition has noticeable impacts on the health, economies, and social structure of developed and developing countries. If aging is viewed as an unalterable decline, the transition will continue to result in negative consequences, such as a growing burden of chronic disease and markets taxed by a flood of dependents that require more social services while paying less into the system.

To soften these negative consequences, attempts should be made to promote healthy aging. This requires an intimate understanding of the biology and physiology of aging. Once aging is more fully understood, population-based or individually-based treatments can be designed to target particular health behaviors or biologic pathways. This may result in the most beneficial health effects and savings on a population level.

Biomarkers of aging are tools which can be used to dissect aging and predict age-associated outcomes in the hopes of promoting healthy aging and longevity. Biomarker research has the potential to "accelerate the discovery of the causes and risk factors associated with disease and disability among older adults" and "bridge basic discovery and intervention development," areas identified as critically important by the National Institute on Aging's strategic plan. Unfortunately, few primary markers of aging have been validated and translated to clinical practice, which is complicated by extrapolating effects from population averaged models into to individuals.

With this research we aimed to determine the association of LTL and lens transparency to markers of aging and disease to advance them as potential biomarkers of aging in humans. These biomarkers were selected for investigation for several reasons. First, both are relatively novel. Thus, future studies will be valuable to the scientific community. Second, these biomarkers are measured in different tissues and may reflect different processes which contribute to aging. Telomeres are part of DNA and are believed to integrate measurement of systemic oxidation, inflammation, and cellular senescence, and may reflect the aging immune system and/or the immune system's interaction with the rest of the body, particularly the vasculature. Transparency is a physical property of the lens that is a function of protein integrity. The proteins that comprise the lens are used throughout the body for the same purpose, to maintain protein conformation, especially under stress, and because few direct markers of the maintenance of protein integrity are available to use in epidemiologic research lens transparency might be particularly valuable for measuring this key aspect of aging. Third, LTL and lens transparency can be measured relatively easily, facilitating clinical translation. LTL is measured using modern molecular techniques such as PCR and Southern blot that are increasingly high-throughput and low cost. Lens transparency can be measured using optical devices repeatedly and accurately, and other alternatives exist, such as grading scales for cataract severity. These theoretical and practical aspects of LTL and lens transparency provide a basis for their investigation as biomarkers of aging and/or age-related disease.

The literature illustrates relatively consistently that LTL is shorter in older vs. younger people, men vs. women, and Caucasians vs. African Americans. Associations with other age-related phenotypes are equivocal. Our data help demonstrate that LTL may be an independent marker of age-related disease burden which may have common causes across tissues, but may be

most strongly associated with cardiovascular disease or white matter disease. What does this new data imply about LTL's relevance to public health? First, the new data implies that LTL may be an intermediate marker for enumerating mechanisms which underlie the pathogenesis of disease across systems. This could aid development of screening tools and interventions to detect and prevent aging and/or disease throughout the body, which would be more efficient than developing select interventions to detect or slow aging or disease in individual systems. Second, it supports previous data which suggests LTL may not be a useful marker for studying or predicting some specific age-related diseases, either because it is weakly associated with these specific diseases or because more accurate and biologically relevant predictors are known for which we already have targeted therapy (e.g., in the case of cardiovascular disease, statin therapy to treat high cholesterol and nicotine replacement therapy to promote smoking cessation). Thus, it helps researchers target their resources. Third, the data highlights that researchers must investigate other parameters of LTL to see if they are predictive of relevant outcomes, such as studying the shortest telomere lengths vs. mean telomere length vs. longitudinal change in telomere length. This may illustrate that certain aspects of telomere dynamics, such as premature shortening or rate of shortening, are more valuable for monitoring aging or disease than mean telomere length. Fourth, we feel that researchers should investigate if telomere length in different tissues is differently associated with aging-related outcomes rather than relying on LTL as a representative marker of telomere lengths. Because telomere length is a fundamental regulator of cellular senescence it seems unwise to disregarding it as a potential biomarker of aging without clarifying these additional points. In the future, if, for example, the rate of telomere shortening is found to correlate strongly with the rate of disease pathogenesis across tissues, the rate of

telomere shortening could serve as a useful first-pass screening tool to illustrate the overall biologic integrity of a human throughout the lifespan.

Previous studies of lens transparency have been focused on the evolution and correlates of age-related cataract, the accuracy and reproducibility of cataract grading scales and lens imaging devices, or changes in lens parameters (e.g., density, width, curvature) with age. Our studies are the first to specifically assess lens transparency vs. cataract surgery as putative biomarkers of aging and/or disease by associating them with LTL, measurements of cardiovascular and metabolic health, cognition and ApoE genotype. Our results suggest that the lens may be a poor indicator of atherosclerosis, but that low lens transparency may be a marker for drastically shorter LTL, diabetes, worse cognition, and ApoE genotype. Of these findings, the most intriguing and relevant to public health is the association with cognition and ApoE genotype. Because there is no effective screening method currently used for Alzheimer's disease, and the pathogenesis of this disease is still unclear, lens transparency may be a promising candidate for clinical translation and mechanistic studies of amyloid-related brain pathology. This may help in detecting brain pathology early or tracking pathology over time. Longitudinal studies correlating changes in lens transparency with changes in cognition and *in vivo* markers of amyloid aggregation may provide strong evidence for use of lens transparency as an indicator of brain health.

Ultimately, the goal of aging research is to determine the biology of aging in order to develop interventions which promote healthy aging and longevity. Although aging is universal, irreversible, and deleterious, it is clear from animal models and human studies that aging can be modulated and that some members of the same species age better than others. In humans it is critical to develop and validate interventions to extend healthspan to ameliorate the negative

effects of aging. Examples of promising interventions undergoing assessment include structured exercise programs (e.g., the LIFE study),[226] caloric restriction regimens (e.g., the CALERIE study),[227] and community-based prevention programs (e.g., 10 Keys to Healthy Aging).[228,229] Once their specificity is more clearly defined, LTL and lens transparency may serve as biomarkers to test the effectiveness of these interventions and others to promote healthy aging and longevity. Because they can be measured over time, LTL and lens transparency may be particularly amenable to clinical translation as markers of health throughout the lifespan.

APPENDIX A

A.1 SUPPLEMENTARY TABLES

Supplementary Table A.1.1: Association of lens transparency at 4th EBT examination to total aortic calcium score at 1st, 2nd, 3rd, and 4th EBT examinations

Total aortic calcium score	Cataract surgery (N=231)		Cataract surgery and transparency (N=136)		Cataract surgery and transparency (N=136)	
	No (N=167)	Yes (N=64)	≥90% Transparent (N=39)	<90% Transparent (N=97) †	≥95% Transparent (N=26)	<95% Transparent (N=110) †
4th EBT (N=200)						
0 (N=8), N (%)	6 (4.0)	2 (4.0)	0 (0.0)	3 (3.8)	0 (0.0)	3 (3.3)
1-99 (N=17), N (%)	13 (8.7)	4 (8.0)	5 (13.2)	5 (6.3)	3 (11.5)	7 (7.6)
100+ (N=175), N (%)	131 (87.3)	44 (88.0)	33 (86.8)	72 (90.0)	23 (88.5)	82 (89.1)
Score, median (IQR)	1783 (3811)	1595 (5455)	1796 (3903)	1721 (4360)	1710 (4232)	1721 (3978)
3rd EBT (N=222)						
0 (N=29), N (%)	22 (13.6)	7 (11.7)	5 (12.8)	10 (10.9)	3 (11.5)	12 (11.4)
1-99 (N=27), N (%)	20 (12.4)	7 (11.7)	6 (15.4)	8 (8.7)	5 (19.2)	9 (8.6)
100+ (N=166), N (%)	120 (74.1)	46 (76.7)	28 (71.8)	74 (80.4)	18 (69.2)	84 (80.0)
Score, median (IQR)	659 (1654)	663 (2476)	891 (1649)	663 (2457)	1053 (2069)	651 (2103)
2nd EBT (N=198)						
0 (N=33), N (%)	22 (15.0)	11 (21.6)	7 (18.4)	14 (17.5)	4 (16.0)	17 (18.3)
1-99 (N=47), N (%)	34 (23.1)	13 (25.5)	7 (18.4)	19 (23.8)	3 (12.0)	23 (24.7)
100+ (N=118), N (%)	91 (61.9)	27 (52.9)	24 (63.2)	47 (58.8)	18 (72.0)	53 (57.0)
Score, median (IQR)	144 (530)	114 (652)	153 (404)	143 (634)	295 (350)	139 (592)
1st EBT (N=225)						
0 (N=60), N (%)	43 (26.5)	17 (27.0)	9 (25.0)	23 (24.0)	7 (29.2)	25 (23.2)
1-99 (N=62), N (%)	43 (26.5)	19 (30.2)	9 (25.0)	27 (28.1)	4 (16.7)	32 (29.6)
100+ (N=103), N (%)	76 (46.9)	27 (42.9)	18 (50.0)	46 (47.9)	13 (54.2)	51 (47.2)
Score, median (IQR)	83 (341)	52 (405)	96 (291)	91 (413)	124 (308)	87 (373)

*P<0.10. †Includes participants who underwent cataract surgery.

Supplemental Table A.1.2: Association of lens transparency at 4th EBT examination to carotid intima-media thickness at 4th EBT examination

	Cataract surgery (N=231)		Cataract surgery and transparency (N=136)		Cataract surgery and transparency (N=136)	
Intima-media thickness (mm)	**No (N=167)**	**Yes (N=64)**	**≥90% Transparent (N=39)**	**<90% Transparent (N=97)** †	**≥95% Transparent (N=26)**	**<95% Transparent (N=110)** †
Mean (SD) of average measurements	0.80 (0.12)	0.81 (0.13)	0.80 (0.13)	0.81 (0.12)	0.82 (0.15)	0.81 (0.12)
Mean (SD) of maximum measurements	1.01 (0.17)	1.04 (0.18)	1.02 (0.18)	1.03 (0.18)	1.03 (0.21)	1.03 (0.17)

*P<0.10

†Includes participants who underwent cataract surgery.

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