TELOMERE LENGTH AS A BIOMARKER OF AGING AND DISEASE

By MATTHEW JOHNATHAN JOSEPH D'MELLO, BHSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree Master of Science

McMaster University

Master of Science (2014)

Health Research Methodology

McMaster University

Hamilton, Ontario

TITLE: Telomere length as biomarker of aging and disease

AUTHOR: Matthew D’Mello, BHSc

SUPERVISOR: Guillaume Pare, MD, MSc

PAGES: xiii, 108

Abstract

Background: Telomeres are repetitive, gene-poor regions that cap the ends of DNA and help to maintain chromosomal integrity. Their shortening is caused by inflammation and oxidative stress within the cellular environment and ultimately leads to cellular senescence. Shortened leukocyte telomere length (LTL) is hypothesized to be a novel biomarker for age-related diseases and may therefore be useful in the prediction of cardiometabolic outcomes above conventional risk factors.

Methods: A systematic review and meta-analysis was undertaken to summarize existing literature on the association between LTL and myocardial infarction (MI), stroke, and type 2 diabetes (T2D). MEDLINE (1966–present), and EMBASE (1980-present) were last searched on September 9th 2013. Studies were combined using the generic inverse variance method and both fixed and random effects models. Additionally, LTL was measured in 3972 MI patients and 4321 controls from an international study on risk factors for MI (INTERHEART), and 8635 participants from an epidemiological study on dysglycemia and T2D (EpiDREAM/DREAM) prospectively followed (approximately 3.5 years) for incident cardiometabolic events.

Results: Based on current literature, a 1-standard deviation decrease in LTL was significantly associated with stroke (OR=1.21, 95% CI=1.06-1.37; I^2=61%), myocardial infarction (OR=1.24, 95% CI=1.04-1.47; I^2=68%), and type 2 diabetes (OR=1.37, 95% CI=1.10-1.72; I^2=91%). Stratification by measurement technique,

study design, study size, and ethnicity explained heterogeneity in certain cardiometabolic outcomes. Within INTERHEART participants, a 1 unit decrease in LTL was associated with an increased risk of MI (OR=2.17, 95% CI=1.74-2.72). Effect estimates were consistent across all ethnic groups (p=0.19). In EpiDREAM a significant association between LTL and T2D or incident cardiometabolic outcomes was not observed.

Conclusion: Telomere length appears to be a marker for MI above conventional risk factors. Further research is needed to explain existing heterogeneity in the literature with respect to LTL and T2D.

Acknowledgments

I would like to first, and foremost, thank my friends and family for their support during my weekends at the lab and late nights trying to learn how to program. In particular, I would like to acknowledge my best friend, Sahaana Rangarajan, who has undoubtedly been the most instrumental to my success through her unwavering companionship.

I would also like to express my gratitude to Dr. Guillaume Pare not only for his mentorship during my Masters and undergraduate thesis, but also for his friendship and ability to create an excellent working environment for all his students. He is the hardest working person I know and an inspiration to future clinician-scientists.

I would like to thank Dr. Gerstein and Dr. Anand for their direction and support with the crafting of my project.

I would also like to thank Dr. Kavsak for his support as an external examiner.

Lastly, I would like to thank all members (past and present) of the genetic and molecular epidemiology laboratory (GMEL) for their friendship and help with various aspects of the project. In particular, I would like to acknowledge Stephanie Ross for providing feedback on manuscript drafts, John Kim and Kripa Raman for teaching me to program in R, Senay Asma for her aid with statistical inquiries, and Michelle Souter for truly helping to make my telomere assay “high-throughput”.

Table of contents

List of Abbreviations

A – Adenine

ApoA –apolipoprotein A1

ApoB – apolipoprotein B

bp – Base pair

BMI – Body mass index

C – Cytosine

CI- Confidence interval

CV – Coefficient of variation

DNA – Deoxyribonucleic acid

dsDNA – Double Stranded DNA

CAD – Coronary artery disease

CHF- Congestive Heart Failure

CVD – Cardiovascular disease

IFG – Impaired fasting glucose

IGT – Impaired glucose tolerance

HbA1c – Glycated hemoglobin

HDL- High density lipoprotein

HR – Hazard Ratio

G – Guanine

LDL – Low density lipoprotein

LTL- Leukocyte telomere length

NO – Nitric Oxide

NOS- Newcastle-Ottawa Scale (NOS)

MACE – Major adverse cardiac event

MI –Myocardial infarction

OGT – Oral glucose tolerance

OR – Odds Ratio

Ram- Ramipril

ROS – Reactive Oxygen Species

Rosi- Rosiglitazone

SD- Standard deviation

ssDNA – Single Stranded DNA

T – Thymine

TL – Telomere length

qPCR- Quantitative polyermase chain reaction

T/S – Telomere to single copy gene ratio

List of Tables

List of Supplementary Tables

List of Figures

List of Supplementary Figures

1. Chapter One: Introduction

1.1. Telomere Structure, Function, & Dynamics

Telomeres are repetitive, gene poor regions located at the ends of each chromosome[1]. Varying in size and composition between species, telomeres play a critical role in providing chromosomal stability. In vertebrates they are composed of hexameric sequences of nucleotides, TTAGGG, which repeat many thousands of times. As such, telomeres are estimated to range in size from 3 to 18 kilobases[2].

Telomeres are mostly double stranded, but at the extreme ends of each telomere exists single-stranded stretch of G-rich DNA, known as the 3' overhang[3]. This section of DNA is remarkably unstable because enzymes within the nucleus recognize single-stranded DNA (ssDNA) as damaged and attempt to repair it through initiating chromosomal fusion – a highly deleterious process[4]. Telomeres, however, effectively hide the 3' overhang through incorporating it back into the double stranded telomeric regions with the aid of stabilizing protein complexes[5]. As depicted in Figure 1.1, Shelterin protein complexes are found throughout the telomeric region and are composed of six subunits. The complexes located distally on the chromosome aid the 3' overhang in folding backwards to form a loop-like structure. Pairing itself with a complementary sequence, the ssDNA 3' overhang now becomes double-stranded and is considered stable.

A critical function of telomeres is preventing gene-rich DNA from being lost during replication. An inherent problem exists in DNA replication whereby the main enzyme responsible for replication (DNA polymerase) cannot copy the extreme ends of chromosomes and as a result each chromosome decreases in length with every round of replication. Known as the end replication problem, this issue exists largely because DNA polymerase requires short strands of complimentary RNA (known as primers) to bind each strand of DNA after it has been unwound in order to initiate the replication process (see Figure 1.2). When replication is complete these primers fall out and another enzyme (DNA ligase) fills in the gaps left behind. DNA ligase requires a 3' end from a preceding nucleotide in order to fill in gaps and since gaps at the end of chromosomes lack a free 3' end, they cannot be replicated[6]. Consequently, chromosomes shorten by approximately the size of a primer (40-200bp) each replication cycle.

Telomeres are essential with respect to protecting against the end replication problem as they provide non-coding DNA which can be lost during the replication process with no adverse effects[1]. Problems in the cell occur when gene rich sections fail to be replicated. However, there are intrinsic cellular mechanisms to prevent this from taking place. When telomeres reach a critical length (~4000bp), they become too short to form the loop that protects the 3' overhang[7]. Consequently, the ssDNA is exposed and cellular checkpoint mechanisms automatically initiate a process (cellular senescence) to halt

replication. If initial checkpoints fail, additional checkpoints initiate apoptosis[8]. Finally, if even further malfunctions persist then chromosomal fusion ensues.

Chromosomal fusion is a cancerous transformation for the cell because chromosomes that fuse often have large sections of DNA deleted, duplicated, or translocated[9]. The instability of one chromosome with short telomeres (and failing checkpoint mechanisms) has the ability to create genome-wide instability. It has been observed that an unstable chromosome goes on to transfer its instability, though fusion processes, to up to six additional chromosomes. Carcinomas have been found in mice deficient in both 53 and enzymes known to maintain telomere length[10]. These mice had notably shorter telomere length as well as an increased number of chromosomal rearrangements..

1.2. Telomeres and Biological Aging

Biological aging is largely characterized by gradual tissue deterioration and loss of function. As opposed to chronological (or calendar) age which measures the amount of time an organism has existed, biological age is used more as an indicator of the physical state of an organism. Biological ageing does not necessarily proceed at the same rate for all individuals. A universal hallmark of biological ageing is the accumulation of cells in the senescent state[11].

Cellular senescence is the process through which a cell ceases to replicate and its homeostatic processes become impaired. Naturally all cells eventually become senescent after they have reached approximately 40-50

divisions[12]. When the senescent cells begin to build up tissues start to deteriorate and lose function. The end replication problem is central to this process because cellular senescence primarily takes place when cells have undergone too many divisions and telomeres can no longer form their protective cap. Cellular processes that directly increase cell cycle progression thus cause premature biological ageing[13].

One cause of accelerated cell cycle progression is a process called oxidative stress. Oxidative stress refers to the imbalance between the creation of reactive oxygen species (ROS) and antioxidant molecules within the cell. ROS are highly reactive molecules containing an oxygen atom with an unpaired electron. Mainly produced by the mitochondria during oxidative phosphorylation, ROS are important for cell signalling and homeostasis, but also have the ability to damage surrounding molecules such as proteins, lipids, and DNA[14]. Increased levels of ROS have recently been shown accelerate the cell cycle through modulating cell proliferation pathways (MAPK and p21) and activating growth factor receptors in the absence of ligands[15].

Chronic inflammation is an additional initiator of cellular senescence[16]. Characterized by mononuclear cell infiltration, tissue destruction, and fibrosis, chronic inflammation is best described as a prolonged low grade inflammation. As an organism ages it is exposed to an increasing number of substances that it perceives as antigens. These antigens are attacked by immune cells which are

attracted to cytokine/chemokines released by cells in distress. Immune cells initiate the production of ROS to eliminate pathogens as well as stimulate tissue repair processes. Released growth factors consequently cause accelerated cell cycle progression.

1.3. Cardiovascular Disease & Telomeres

Cardiovascular disease is broad term used to classify all diseases affecting the heart and blood vessels. The main components of CVD are coronary heart disease (angina and myocardial infarction) and cerebrovascular disease (stroke). CVD is primarily caused by the buildup of cholesterol plaque along the walls of arteries. This gradual accumulation of fatty substances and cholesterol slowly hardens and narrows arteries supplying blood to the heart or brain. Plaques are vulnerable to rupture which then triggers blood clot formation (thrombosis) and subsequently full artery occlusion[17].

Even after taking into account all other major risk factors, age is the most significant predictor for CVD. As individuals age their vascular tissue begins to deteriorate and the regenerative capacity is impaired. This is thought to arise from a depletion of vascular progenitor cell reserves and the accumulation of senescent endothelial and vascular smooth muscle cells. These senescent cells display impaired nitric oxide (NO) production and increased expression of cytokines/chemokine which together create a pro-inflammatory/pro-thrombotic environment[18]. The observation of senescent cells and a pro-inflammatory

environment within atherosclerosis suggests a potential link between telomere length and CVD.

Indeed, laboratory studies provide evidence for a link between telomere length and CVD. In cultured aortic endothelial cells, the inhibition of telomere activity has been shown to increase the production of ROS, decrease NO levels, and increase expression of inflammatory adhesion molecules[19]. Moreover, vascular tissue biopsies of patients with atherosclerosis display shortened telomere length compared to biopsies from healthy controls[20]. Interestingly, clinical evidence for an association remains controversial. Associations between telomere length and risk factors for CVD such as obesity, smoking, hypertension, and diabetes are mixed[21,22]. Likewise, associations with CVD outcomes such as myocardial infarction, stroke, and congestive heart failure are inconsistent in the literature[23–27]. Small sample sizes, inadequate telomere measurement, and poor sampling are all suggested reasons for discrepancies[28].

1.4. Type 2 Diabetes & Telomeres

Type 2 diabetes (T2D) is a chronic disease characterized by high levels of blood glucose (hyperglycemia) in the context of insufficient production of insulin by pancreatic beta-cells and relative resistance to insulin of tissues[29]. Beta cell dysfunction is often cited as the essential factor in the initiation and progression of T2D. This dysfunction is postulated to be caused by increased levels of both glucose and free fatty acids (glucolipotoxicity)[30]. Heightened levels of these

substances accelerate the metabolic rate of the mitochondria and stimulate ER stress which in turn increases ROS production and protein misfolding, respectively[31].

Similar to CVD, age is clearly a strong risk factor for T2D. The poor functioning of tissues such as those that are made up of beta cells in the pancreas is typically observed in individuals of advanced age. The chronic exposure to hyperglycemia and free fatty acids over time leads to a gradual accumulation of senescent and apoptotic beta cells which then form the basis for transitioning from a normal glycemic state to dysglycemia and then eventually to T2D[32]. Given that telomeres play a mediating role in inducing senescence there is a plausible link between telomere length and T2D.

Current literature provides varying support for an association between telomere length and diabetes. Mouse models have shown that inhibition of telomere loss results in improved glucose homeostasis[33]. In smaller clinical studies, telomere length in patients with impaired glucose tolerance was found to be longer than T2D patients, but shorter than healthy controls[34]. In larger studies, however, the association between telomere length and T2D is inconsistent. Prospective studies in particular are extremely rare and therefore the ability of telomeres to predict future incident T2D remains controversial.[35,36]. The discordant conclusions reported are difficult to reconcile, but likely causes are similar to those impacting CVD literature.

1.5. A Common Mechanism

Many hypotheses exist that attempt to explain the link between CVD and T2D. In particular, the 'common soil hypothesis' implies that susceptibility to both conditions arises from genetic predisposition coupled with unique environmental conditions[37]. Support for this hypothesis is found in the fact that many risk factors for T2D are additionally risk factors for CVD such as high triglycerides, low high density lipoprotein, and hypertension[38].

At the cellular level both CVD and T2D are initiated by complex mechanisms that participate in cross-talk. Chronic inflammation and oxidative stress leading to cellular senescence and apoptosis are critical pathways linking insulin resistance with dysfunction of beta cells and vascular endothelium. Indeed, these pathways are characterized by increased ROS in the context of diminished antioxidants[39,40]. Hence, a relative imbalance between ROS and antioxidants may be the root cause of biological ageing and the common tie between CVD and T2D.

Chronological age is strong risk factor for both CVD and diabetes, but great inter-individual variability still remains unexplained with respect to disease onset and progression. Biological age may in fact account for this as it reflects individual differences in genetic makeup and environmental exposures stress. Given that telomere length is inversely related to chronic inflammation and

oxidative stress associated with biological ageing, it may serve as a useful biomarker for both CVD and T2D.

1.6. Objectives

Section I:

- To systematically review and meta-analyze reported associations between telomere length and cardiometabolic outcomes in current literature
- To identify and explore sources of heterogeneity in effect estimates
- To assess the overall strength of evidence supporting telomere length as a biomarker of cardiometabolic outcomes.

Section II:

- To evaluate the use of telomere length as biomarker of MI and T2D in INTERHEART and EpiDREAM studies
- To identify/validate factors influencing telomere attrition
- To assess whether associations are consistent across different ethnic groups

1.7. Hypotheses

Section I

- I hypothesize that a shorter telomere length will be associated with all cardiometabolic outcomes (MI, T2D, and Stroke) investigated and that

heterogeneity likely will be explained by differences in sample sizes and laboratory techniques.

Section II

- I hypothesize that shorter telomere length will be associated with T2D and MI and that effect estimates will not vary based on ethnicity.

1.8. References

1. Blackburn, E. H. Structure and function of telomeres. *Nature* **350,** 569–73 (1991).

2. Moyzis, R. & Buckingham, J. A highly conserved repetitive DNA sequence,(TTAGGG) n, present at the telomeres of human chromosomes. *Proc. …* **85,** 6622–6626 (1988).

3. Wright, W. E., Tesmer, V. M., Huffman, K. E., Levene, S. D. & Shay, J. W. Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes Dev.* **11,** 2801–2809 (1997).

4. Hande, M. P., Samper, E., Lansdorp, P. & Blasco, M. A. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. *J. Cell Biol.* **144,** 589–601 (1999).

5. De Lange, T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* **19,** 2100–10 (2005).

6. Levy, M. Z., Allsopp, R. C., Futcher, A. B., Greider, C. W. & Harley, C. B. Telomere end-replication problem and cell aging. *J. Mol. Biol.* **225,** 951–60 (1992).

7. Allsopp, R. & Harley, C. Evidence for a critical telomere length in senescent human fibroblasts. *Exp. Cell Res.* (1995). at <http://www.sciencedirect.com/science/article/pii/S001448278571213X>

8. d'Adda di Fagagna, F. *et al.* A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426,** 194–8 (2003).

9. Bailey, S. M., & Murnane, J. P. (2006). Telomeres, chromosome instability and cancer. *Nucleic acids research*, *34*(8), 2408-2417.

10. Chang, S., Khoo, C., & DePinho, R. A. (2001, June). Modeling chromosomal instability and epithelial carcinogenesis in the telomerase-deficient mouse. In*Seminars in cancer biology* (Vol. 11, No. 3, pp. 227-239). Academic Press.

11. Goldstein, S. Replicative senescence: the human fibroblast comes of age. *Science (80-.).* **249,** 1129–1133 (1990).

12. Shay, J. W. & Wright, W. E. Hayflick, his limit, and cellular ageing. *Nat. Rev. Mol. Cell Biol.* **1,** 72–6 (2000).

13. Speakman, J. R. & Selman, C. The free-radical damage theory: Accumulating evidence against a simple link of oxidative stress to ageing and lifespan. *Bioessays* **33,** 255–9 (2011).

14. Circu, M. L. & Aw, T. Y. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **48,** 749–62 (2010).

15. Verbon, E. H., Post, J. A. & Boonstra, J. The influence of reactive oxygen species on cell cycle progression in mammalian cells. *Gene* **511,** 1–6 (2012).

16. Freund, A., Orjalo, A. V, Desprez, P.-Y. & Campisi, J. Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol. Med.* **16,** 238–46 (2010).

17. Campbell, G. R. & Campbell, J. H. Smooth muscle phenotypic changes in arterial wall homeostasis: Implications for the pathogenesis of atherosclerosis. *Exp. Mol. Pathol.* **42,** 139–162 (1985).

18. Formato, M. *et al.* Evidence for a proinflammatory and proteolytic environment in plaques from endarterectomy segments of human carotid arteries. *Arterioscler. Thromb. Vasc. Biol.* **24,** 129–35 (2004).

19. Minamino, T. *et al.* Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* **105,** 1541–4 (2002).

20. Nzietchueng, R. *et al.* Telomere length in vascular tissues from patients with atherosclerotic disease. *J. Nutr. Health Aging* **15,** 153–6 (2011).

21. Sampson, M. J., Winterbone, M. S., Hughes, J. C., Dozio, N. & Hughes, D. a. Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes. *Diabetes Care* **29,** 283–9 (2006).

22. Shen, Q. *et al.* Association of leukocyte telomere length with type 2 diabetes in mainland Chinese populations. *J. Clin. Endocrinol. Metab.* **97,** 1371–4 (2012).

23. Brouilette, S., Singh, R. K., Thompson, J. R., Goodall, A. H. & Samani, N. J. White cell telomere length and risk of premature myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* **23,** 842–6 (2003).

24. Willeit, P. *et al.* Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. *Arterioscler. Thromb. Vasc. Biol.* **30,** 1649–56 (2010).

25. Weischer, M. *et al.* Short telomere length, myocardial infarction, ischemic heart disease, and early death. *Arterioscler. Thromb. Vasc. Biol.* **32,** 822–9 (2012).

26. Perez-Rivera, J.-A. *et al.* Prognostic value of telomere length in acute coronary syndrome. *Mech. Ageing Dev.* **133,** 695–7 (2012).

27. Zee, R. & Castonguay, A. Relative leukocyte telomere length and risk of incident ischemic stroke in men: A prospective, nested case-control approach. *Rejuvenation …* (2010). at <http://online.liebertpub.com/doi/abs/10.1089/rej.2009.0975>

28. Mather, K. A., Jorm, A. F., Parslow, R. A. & Christensen, H. Is telomere length a biomarker of aging? A review. *J. Gerontol. A. Biol. Sci. Med. Sci.* **66,** 202–13 (2011).

29. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **33 Suppl 1,** S62–9 (2010).

30. Poitout, V. *et al.* Glucolipotoxicity of the pancreatic beta cell. *Biochim. Biophys. Acta* **1801,** 289–98 (2010).

31. Muoio, D. M. & Newgard, C. B. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **9,** 193–205 (2008).

32. Sone, H. & Kagawa, Y. Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia* **48,** 58–67 (2005).

33. Kuhlow, D. *et al.* Telomerase deficiency impairs glucose metabolism and insulin secretion. *Aging (Albany. NY).* **2,** 650–8 (2010).

34. Adaikalakoteswari, A., Balasubramanyam, M., Ravikumar, R., Deepa, R. & Mohan, V. Association of telomere shortening with impaired glucose tolerance and diabetic macroangiopathy. *Atherosclerosis* **195,** 83–9 (2007).

35. Zhao, J., Zhu, Y., Lin, J. & Matsuguchi, T. Short Leukocyte Telomere Length Predicts Risk of Diabetes in American Indians: The Strong Heart Family Study. *Diabetes* 1–34 (2013). at

<http://diabetes.diabetesjournals.org/content/early/2013/08/12/db13-0744.short>

36. You, N.-C. Y. *et al.* A prospective study of leukocyte telomere length and risk of type 2 diabetes in postmenopausal women. *Diabetes* **61,** 2998–3004 (2012).

37. Stern, M. P. Diabetes and cardiovascular disease. The "common soil" hypothesis. *Diabetes* **44,** 369–74 (1995).

38. Smith, S. C. Multiple risk factors for cardiovascular disease and diabetes mellitus. *Am. J. Med.* **120,** S3–S11 (2007).

39. Salpea, K. D. *et al.* Association of telomere length with type 2 diabetes, oxidative stress and UCP2 gene variation. *Atherosclerosis* **209,** 42–50 (2010).

40. Lahera, V. *et al.* Endothelial dysfunction, oxidative stress and inflammation in atherosclerosis: beneficial effects of statins. *Curr. Med. Chem.* **14,** 243–8 (2007).

1.9. Tables & Figures

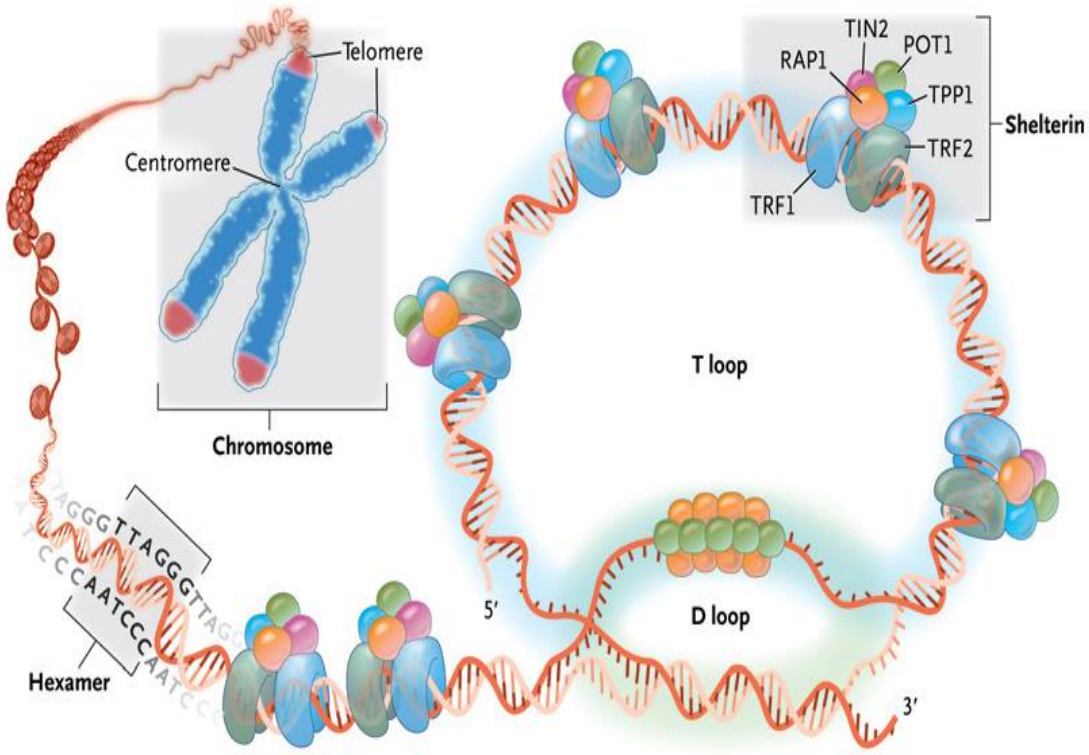


Figure 1.1 Location, Structure, and Composition of Telomeres. Telomeres are located at the end of the chromosome. The hexameric repeats are bound by Shelterin protein complexes. The 3' end of the telomere is ssDNA. It folds back to incorporate itself into the double stranded DNA with the aid of the Shelterin protein complexes.

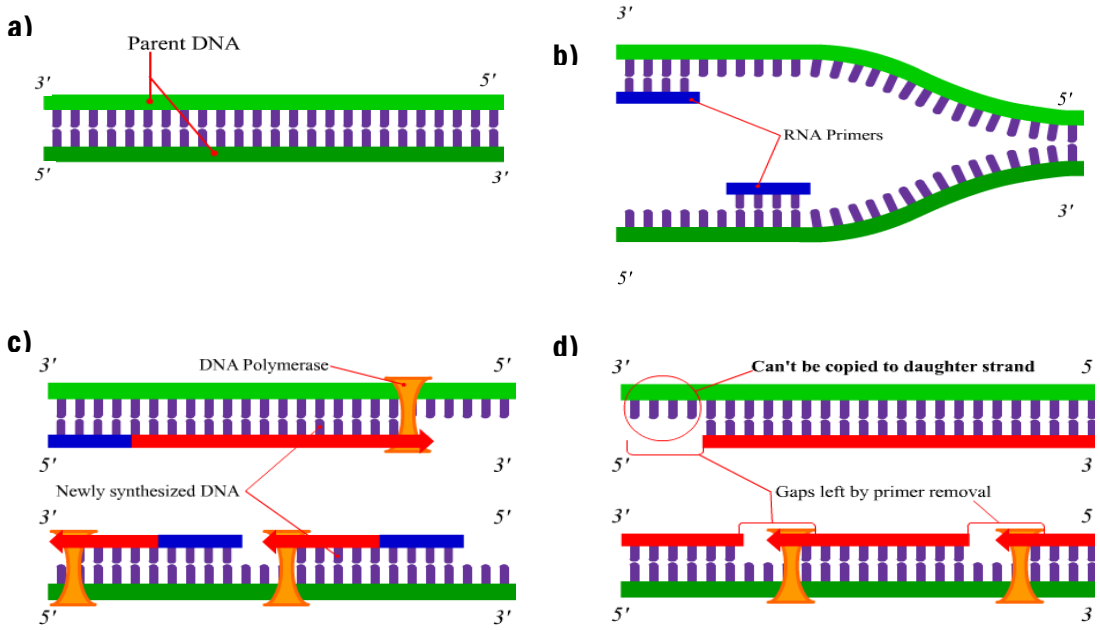


Figure 1.2 The End Replication Problem. A) dsDNA prior to replication. B) dsDNA is unwound and RNA primers bind throughout both template strands. C) DNA polymerase replicates DNA in the 5' to 3' direction, starting at the 3' end of the primer. D) RNA primers are removed and gaps remain. The gap at the start of the DNA strand cannot be filled by DNA ligase because there is no free 3' end.

2. Chapter Two: Leukocyte Telomere Length as a Biomarker for Cardiometabolic Outcomes: A Systematic Review and Meta-Analysis

2.1. Introduction

Telomeres are gene-poor, repetitive TTAGGG nucleotide sequences that cap the ends of chromosomes[1]. Folding back on themselves to form a protective loop, they help stabilize chromosomes through preventing degradation, end-to-end fusion, and abnormal recombination of DNA strands.[2] With each cell cycle telomeres shorten 30-200 nucleotides.[3] This process is further accelerated as a result of oxidative stress and chronic inflammation[4,5]. After telomeres decrease in size to a critical length, they are no longer able to serve their protective purposes. Consequently, cell cycle arrest (senescence) or apoptosis is activated[6]. Since increased cellular senescence and oxidative stress are both key indicators of aging, it has been recently suggested that a shortened average telomere length could serve as a biomarker for aging and age-related diseases[7].

Cardiovascular disease (CVD) and type 2 diabetes (T2D) are two disorders clearly related to age and a reduced life span[8]. The incidence of these cardiometabolic outcomes demonstrates great inter-individual variability within the same age group, suggesting that chronological age is not a precise measure of health status[9]. There is therefore great utility in identifying a biomarker that could provide further information about one's cardiometabolic health in addition to (or in place of) chronological age, as it would aid in both the prediction and prevention of disease[10]. Telomere length may be one such biomarker.

To date, studies of the association between leukocyte telomere length (LTL) which reflects telomere length throughout the body[11] and cardiometabolic outcomes have yielded conflicting results [12–37]. For example, some studies have reported a significant association between telomere length and stroke [16,30], whereas others have failed to demonstrate any such association[27,35]. This inconsistency, which is also seen in studies investigating other cardiometabolic outcomes, indicates that individual studies may not be statistically powered to detect true associations because of inadequate sample sizes. Furthermore, unstandardized laboratory techniques, different study designs, and ethnic diversity within study patient populations have also been suggested as plausible explanations for heterogeneous results[38,39].

The primary objective of this systematic review and meta-analysis is to provide insight into the use of LTL as a biomarker of aging through a comprehensive assessment of the relationship between shortened LTL and the cardiometabolic outcomes of stroke, myocardial infarction (MI), and T2D. Secondary outcomes investigated include CVD related death and a major adverse cardiac event (MACE) composite outcome.

2.2. Methods

Eligibility Criteria

Articles deemed eligible for inclusion into the systematic review reported on the association between LTL and one or more of the following outcomes:

stroke, MI, CVD related death, T2D, or MACE composite. MACE was defined as stroke, MI, or CVD related death. Both cross-sectional and prospective studies were selected for inclusion. If multiple publications reported the same outcome in identical populations, only the most recent publication was included. Publications were excluded if telomere length was not measured in leukocytes. No restrictions were placed on sample size, language of publication, date of publication, or publication status.

Information Sources & Search Strategy

Articles were accessed through OVID from the MEDLINE (1966-present) and EMBASE (1980-present) electronic databases. Limitations on the search restricted citations to only those including humans. Key MeSH terms used in the search strategy included: telomere, myocardial infarction, stroke, diabetes mellitus, and death. See Tables I and II in online-only Data Supplement for complete search strategy for both databases. The last search was run on Sept 9th 2013. To identify further citations, the reference lists of articles retrieved were also hand searched.

Study Selection, Data Collection, & Data Items

Two reviewers, MD and SR, independently selected studies for full text review through title and abstract screening of citations retrieved from all sources. Full-text screening for final inclusion into the systematic review was also carried out independently by both reviewers. Cohen's unweighted kappa was used to

evaluate agreement between both reviewers at each screening stage, and disagreements were resolved through consensus.

A data abstraction sheet was designed and piloted with ten randomly selected studies. MD and SR independently extracted data pertaining to: 1) study type, 2) patient baseline characteristics, 3) LTL measurement technique, 4) study quality indicators, and 5) odds ratio (OR) or hazard ratio (HR) and the associated 95% confidence interval (CI). Disagreements were resolved through consensus.

Risk of Bias in Individual Studies

Risk of bias was independently assessed at the outcome level using an adapted version of the Newcastle-Ottawa Scale (NOS)[40]. Briefly, case-control and cohort studies were scored in 3 separate categories: selection, comparability, and exposure/outcome. Overall each study received a rating from 0-8 stars depending on the likelihood of bias. *A priori* we established that 0-3, 4-6, and 7-8 stars would be considered at high, moderate, and low risk of bias, respectively.

Summary Measures, Synthesis of Results, & Risk of Bias Across Studies

The main summary measure was the pooled OR and 95% CI of a cardiometabolic outcome per standard deviation (SD) decrease in LTL. Cardiometabolic outcomes are relatively rare events and as such we treated HRs as approximates of ORs. As described in the online-only Data Supplement

Methods section, an effort was made to convert to per-SD decrease when associations were reported based on quantile comparisons of LTL (i.e. shortest versus longest quantile). Only the most adjusted effect measures were used so as to account for confounding.

The pooled OR was computed using the generic inverse variance method. Heterogeneity was assessed using the Cochran Q test and considered to be significant if $P<0.05$. Additionally, I^2 was used as a measure of the portion of total variation in estimates that was due to heterogeneity. High heterogeneity was defined as I^2 above 50%, whereas moderate and low heterogeneity were defined as below 50% and 25%, respectively. Pooled summary estimates were initially calculated using the fixed effect model, however if significant heterogeneity was observed a random effects model was alternatively employed. To assess for publication bias across studies inverted funnel plots were created for each outcome and visually inspected for asymmetry. *A priori* subgroup analyses based on study type, LTL measurement technique, sample size, and population ethnicity were conducted to examine possible sources of heterogeneity. Sensitivity analyses, also specified *a priori*, were conducted to observe the impact of removing studies at high or moderate risk of bias, and studies utilizing highly variable LTL measurement techniques (inter-assay coefficient of variation [CV] >10%). Statistical analyses were conducted using Review Manager (v5.2). All reported *P* values were two-sided.

2.3. Results

Selection & Characteristics of Included Studies

As shown in Figure 2.1, the electronic database search of MEDLINE and EMBASE resulted in the identification of 3382 relevant citations. 2112 records remained after duplicate citations were removed and 2023 of these were excluded after title and abstract review for not meeting inclusion criteria. The full text review of the 89 remaining articles yielded 26 publications for inclusion into the systematic review. No additional citations were retrieved from searching reference lists. Key reasons for exclusion included: no cardiometabolic outcomes measured (59), telomere length not obtained from leukocytes (2), and multiple publications of the same data set (2). A Cohen's unweighted kappa of 0.83 was achieved signifying good agreement between both reviewers. Among the 26 included publications, one was excluded from quantitative meta-analysis due to not providing enough information to calculate an appropriate OR and 95% CI. When necessary, authors of included studies were contacted for further information with respect to study characteristics or reported results. One response was received[14].

Tables 2.1-2.3 describe the characteristics of included studies assessing stroke, MI, and T2D. Studies assessing CVD related death and MACE are presented in supplementary Tables 2.3-2.4. One publication consisted of both a case-control and cohort study and therefore a total of 14 case-control and 12

cohort studies were included into the meta-analysis[13]. Participants from the Cardiovascular Health Study and the Physicians Health Study were both included in multiple publications, however different outcomes were reported [16, 17, 34, 35]. A number of studies investigated LTL in specific ethnic groups. European Caucasian was the ethnic group predominantly reported, followed by Asian. Only one study presented effect measures stratified based on different ethnicities[32]. All studies enrolled a similar amount of males and females, except for five that were sex specific[19, 27, 32, 34, 35]. Quantitative polymerase chain reaction (qPCR) was the primary method of telomere measurement, with four studies using the Southern blot technique [11, 15, 16, 17]. Reported mean CVs ranged from 1.3% to 22%. Each study adjusted their reported effect measure for a variety of confounding variables and these are described in the Supplementary Tables 2.5-2.9.

Risk of Bias Within Studies

The risk of bias assessment is presented at the outcome level in Tables 2.1-2.3. The majority of studies included had a low risk of bias according to the NOS quality score. Two studies did not adjust for age in the experimental design and/or analysis[22,24] and thus were considered at risk of bias given the strong relationship between LTL and age. Lack of blinding of laboratory technicians and the use of highly specific patient populations were considered as further sources of bias.

Primary Outcomes: STROKE, MI, & T2D

A consistent positive association between per-SD decrease in LTL and all three primary cardiometabolic outcomes was observed (Figure 2.2). The ten studies reporting stroke had a pooled OR of 1.21 (95% CI=1.06-1.37) and displayed significant heterogeneity (I^2 = 61%, P<0.01) when meta-analyzed. A more modest summary OR was identified when combining the six studies that reported on MI (OR=1.24, 95% CI = 1.04-1.47). A high level of heterogeneity was also detected between these studies (I^2 = 68%, P<0.01). The largest effect size was observed with respect to T2D (OR=1.37, 95% CI = 1.10-1.72). Significant heterogeneity was detected (I^2 = 91%, P<0.01) amongst the seven studies meta-analyzed. The one study not included into the quantitative meta-analysis reported an association between a decrease in LTL and T2D (OR=1.24, 95% CI 1.09-1.42) [23].

Secondary Outcomes: CVD Death & MACE Composite

A consistent positive association between per-SD decrease in LTL and both secondary cardiometabolic outcomes was identified (Supplementary Figures 2.1-2.2). With respect to CVD death, six studies were combined to obtain a pooled OR that reached significance (OR=1.11, 95% CI = 1.00-1.22; I^2 = 29). Three studies reported a MACE composite and when meta-analyzed a significant association was observed (OR=1.14, 95% CI = 1.02-1.29). A high level of heterogeneity was present (I^2 = 64%).

Subgroup Analysis

Subgroup analysis by measurement technique, study type, study size, and ethnicity are presented for each primary outcome in Figure 2.3. Stratifying by qPCR or Southern blot explained some of the heterogeneity in the association between shortened LTL and stroke. I^2 decreased from 61% overall to 35% for studies using Southern blot and 58% for studies using qPCR (P=0.03 for subgroup differences).

Stratifying by study design explained the high level of heterogeneity within the MI (P=0.01 for subgroup differences) and stroke (P=0.03 for subgroup differences) meta-analyses. With respect to MI, case-control studies had a low level of heterogeneity (I^2=0%) whereas cohort studies had a moderate level (I^2=45%). Only the case-control subgroup stayed significantly associated with a shortened LTL after stratification. Within the stroke meta-analysis, case-control studies (I^2=66%) and cohort studies (I^2=56%) remained at a high level of heterogeneity. The cohort subgroup was no longer significantly associated with a shortened LTL after stratification.

Study size subgroup differences were also observed in the MI pooled assessment (P<0.01). The high level of heterogeneity within the pooled OR for MI (I^2 =85%) was almost completely eliminated when stratifying studies by 0-499, 500-999, and >1000 participants (I^2 of 0%, 0%, and 37%, respectively). The association with shortened LTL remained in the two smaller subgroups.

Significant subgroup differences were not observed when stratifying by ethnicity.

Assessment of Publication Bias & Sensitivity Analysis

Publication bias was assessed for all outcomes through visually inspecting asymmetry in the funnel plots presented in Figure 2.4. The funnel plots for both MI and T2D demonstrated moderate asymmetry indicating publication bias. There was little evidence to suggest publication bias with respect to stroke and secondary outcomes (supplementary Figures 2.3-2.4). Removing studies at high risk of bias according to the overall NOS quality score did not significantly alter the associations in any of the cardiometabolic outcomes of interest. Similarly, removing studies not reporting a CV (or reporting a CV>10%) had no significant effect on any pooled ORs (Supplementary Table 2.10).

2.4. Discussion

In this systematic review and meta-analysis of 26 observational studies, a constant positive association with per-SD decrease in LTL was observed across all 5 cardiometabolic outcomes assessed. The main strength of this review lies in the fact that it is a pooled analysis of LTL and cardiometabolic outcomes, thus providing the greatest power to detect associations missed by smaller individual studies. Furthermore, the large number of outcomes assessed within this review allows for a more comprehensive evaluation of LTL as a general biomarker of aging.

MI is a consequence of interrupted blood flow to the heart subsequently leading to the death of cardiomyocytes[41]. Likewise, stroke is characterized by the sudden loss of blood supply to the brain resulting in neuronal death[42]. Both cardiometabolic outcomes are often caused by the formation of unstable atherosclerotic plaques over time within vascular tissue. It has been shown that plaques form as a product of impaired endothelial repair and vessel remodeling, high cell turnover, increased oxidative stress, and up-regulation of inflammatory factors[43]. Interestingly, these plaque formation processes have all been shown to be associated with decreased telomere length in vascular cells[10]. When considered with the fact that LTL is highly correlated with vascular tissue telomere length, it is reasonable to expect shortened LTL in patients at risk of stroke or MI. Evidence from our meta-analysis aligns directly with this hypothesis as we have found that a per-SD decrease in LTL confers a higher risk for both stroke (OR=1.21, 95% CI=1.06-1.37) and MI (OR=1.24, 95% CI=1.04-1.47).

CVD related death had the smallest effect size, despite all other cardiometabolic outcomes demonstrating significant associations with a shortened LTL. A possible explanation for this is a lack of statistical power due to a relatively low number of events observed. Additionally, the age of participants studied may have diminished the pooled estimate as it has been reported that LTL is a poor predictor of survival in elderly individuals (>75 years old)[44]. Two studies included in the meta-analysis used populations with a mean age >75[19,22].

An interesting result was the significant association between a shortened LTL and the MACE composite outcome, suggesting that patients suffering any events due to general cardiovascular aging had a shorter LTL. This effect has been observed and quantified in a study where it was shown that LTL in coronary artery disease (CAD) patients was similar to that of healthy controls which were 11 years older[12].

T2D is a metabolic disorder characterized by increased blood glucose levels due pancreatic beta cell dysfunction in the context of increased insulin requirements[42]. Since T2D diabetes is a strong predictor for CVD, it has been hypothesized that a common biological pathway based on tissue aging and senescence could potentially link the two diseases[45]. Our findings of a significant relationship between a shortened LTL and T2D provide evidence for this hypothesis. Further support of this relationship can be seen in studies reporting cardiovascular events in diabetic patients. For instance, it has been shown that T2D patients with MI have a shorter LTL when compared with T2D controls[25].

Heterogeneity was observed in some of the subgroup analyses. Stratifying by measurement technique, study type, study design, and ethnicity did not produce consistent results across all primary outcomes. As compared to qPCR, the use of Southern blot was associated with a modestly stronger effect estimate for stroke but not for MI. A plausible explanation is increased measurement error associated with qPCR biased the effect estimate towards the

null, although sample size was also smaller in studies using Southern blot. A subgroup difference between study designs in the MI meta-analysis was also observed. Only the case-control subgroup remained significant (OR = 1.51; 95% CI = 1.25-1.82) after stratification suggesting that reverse causation or other biases might inflate risk estimates. This is in contrast to evidence from a recent genome-wide meta-analysis which supports a causal role for LTL shortening in the manifestation of CAD[46]. Finally, study size was inversely correlated with strength of association for T2D and MI, indicating potential publication bias.

Limitations

Some limitations exist to the results presented in this meta-analysis. First, reporting of LTL as a variable differed between many studies and consequently statistical techniques were used to standardize reported effect measures to per-SD decrease. These statistical methods are most accurate for converting from LTL categorized as an ordinal variable (tertiles or quartiles) when a linear trend with respect to increasing effect size is observed across quantiles. Most studies included in this meta-analysis demonstrate this trend, but due to smaller sample sizes some studies deviate from it. Moreover, based on the assumption that LTL is a true biomarker for aging, the inclusion of effect measures adjusted for chronological age likely attenuates the strength of association with cardiometabolic outcomes. With the exception of two, all studies included in this

meta-analysis adjusted for age and as such our findings are likely underestimates of underlying associations.

Lastly, ethnic subgroup analysis was hindered due to lack of reporting of ethnicity specific effect measures. Some studies included into the meta-analysis utilized multi-ethnic populations and adjusted for this in their analysis, but did not report ethnic-specific estimates. These authors were contacted for further information regarding ethnic group specific effect measures, but no response was received. Although limited, we carried out the subgroup analysis including a mixed group to represent studies reporting adjusted analysis for multi-ethnic populations.

2.5. Conclusion

We present a systematic review and meta-analysis evaluating the use of LTL as a biomarker for aging through its association with age-related cardiometabolic outcomes. Despite a significant association between per-SD decrease in LTL and all outcomes measured, the results from this meta-analysis should be interpreted carefully as the observed heterogeneity is yet to be fully explained. Larger observational studies, with well characterized patient populations and reliable LTL measurement techniques, are required to further explore sources of heterogeneity and ultimately validate the use of LTL as a marker for biological age.

2.6. References

1. Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, et al. Jones MD. A highly conserved repetitive DNA sequence,(TTAGGG) n, present at the telomeres of human chromosomes. PNAS. 1988; 85:6622-6

2. Ducray C, Pommier JP, Martins L, Boussin FD, Sabatier L. Telomere dynamics, end-to-end fusions and telomerase activation during the human fibroblast immortalization process. Oncogene. 1999; 18:4211–23.

3. Harley C, Futcher A, Greider C. Telomeres shorten during ageing of human fibroblasts. Nature. 1990; 345:458-60

4. Oikawa S, Kawanishi S. Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett. 1999; 453:365–8.

5. O'Donovan A, Pantell MS, Puterman E, Dhabhar FS, Blackburn EH, Yaffe K, et al. Cumulative inflammatory load is associated with short leukocyte telomere length in the Health, Aging and Body Composition Study. PLoS One. 2011; 6:e19687.

6. Allsopp R, Harley C. Evidence for a critical telomere length in senescent human fibroblasts. Exp. Cell Res. 1995; 219:130-136

7. Sanders JL, Newman AB. Telomere Length in Epidemiology: A Biomarker of Aging, Age-Related Disease, Both, or Neither? Epidemiol. Rev. 2013; 35:112–131.

8. Kannel WB, McGee DL. Diabetes and cardiovascular disease. JAMA. 1979; 241: 2035-8.

9. Halvorsen TL, Beattie GM, Lopez AD, Hayek A, Levine F. Accelerated telomere shortening and senescence in human pancreatic islet cells stimulated to divide in vitro. J. Endocrinol. 2000; 166:103–9.

10. Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A, et al. Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. Circ. Res.. 2006; 99:156–64.

11. Ahmad S, Heraclides A, Sun Q, Elgzyri T, Rönn T, Ling C, et al. Telomere length in blood and skeletal muscle in relation to measures of glycaemia and insulinaemia. Diabet. Med. 2012; 29:377–81.

12. Brouilette S, Singh RK, Thompson JR, Goodall AH, Samani NJ. White cell telomere length and risk of premature myocardial infarction. Arterioscler. Thromb. Vasc. Biol. 2003; 23:842–6.

13. Ding H, Chen C, Shaffer JR, Liu L, Xu Y, Wang X, et al. Telomere length and risk of stroke in Chinese. Stroke. 2012; 43:658–63.

14. Epel ES, Merkin SS, Cawthon R, Blackburn EH, Adler NE, Pletcher MJ, et al. The rate of leukocyte telomere shortening predicts mortality from cardiovascular disease in elderly men. Aging. 2009; 1:81–8.

15. Farzaneh-Far R, Cawthon RM, Na B, Browner WS, Schiller NB, Whooley MA. Prognostic value of leukocyte telomere length in patients with stable coronary artery disease: data from the Heart and Soul Study. Arterioscler. Thromb. Vasc. Biol. 2008; 28:1379–84.

16. Fitzpatrick AL, Kronmal RA, Kimura M, Gardner JP, Psaty BM, Jenny NS, et al. Leukocyte telomere length and mortality in the Cardiovascular Health Study. J. Gerontol. A. Biol. Sci. Med. Sci. 2011; 66:421–9.

17. Fitzpatrick AL, Kronmal RA, Gardner JP, Psaty BM, Jenny NS, Tracy RP, et al. Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. Am. J. Epidemiol. 2007; 165:14–21.

18. Fyhrquist F, Silventoinen K, Saijonmaa O, Kontula K, Devereux RB, de Faire U, et al. Telomere length and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: the LIFE study. J. Hum. Hypertens. 2011; 25:711–8.

19. Houben JMJ, Giltay EJ, Rius-Ottenheim N, Hageman GJ, Kromhout D. Telomere length and mortality in elderly men: the Zutphen Elderly Study. J. Gerontol. A. Biol. Sci. Med. Sci. 2011; 66:38–44.

20. Hovatta I, de Mello VDF, Kananen L, Lindström J, Eriksson JG, Ilanne-Parikka P, et al. Leukocyte telomere length in the Finnish Diabetes Prevention Study. PLoS One. 2012; 7:e34948.

21. Jiang X, Dong M, Cheng J, Huang S, He Y, Ma K, Tang B, Guo Y. Decreased leukocyte telomere length (LTL) is associated with stroke but unlikely to be causative. PLoS One. 2013; 8:e68254.

22. Martin-Ruiz CM, Gussekloo J, van Heemst D, von Zglinicki T, Westendorp RGJ. Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population-based study. Aging Cell. 2005; 4:287–90.

23. Monickaraj F, Aravind S, Gokulakrishnan K, Sathishkumar C, Prabu P, Prabu D. Accelerated aging as evidenced by increased telomere shortening and mitochondrial DNA depletion in patients with type 2 diabetes. Mol. Cell. Biochem. 2012; 365:343–50.

24. Njajou OT, Hsueh W-C, Blackburn EH, Newman AB, Wu S-H, Li R, et al. Association between telomere length, specific causes of death, and years of healthy life in health, aging, and body composition, a population-based cohort study. J. Gerontol. A. Biol. Sci. Med. Sci. 2009; 64:860–4.

25. Olivieri F, Lorenzi M, Antonicelli R, Testa R, Sirolla C, Cardelli M, et al. Leukocyte telomere shortening in elderly Type2DM patients with previous myocardial infarction. Atherosclerosis. 2009; 206:588–93.

26. Salpea KD, Talmud PJ, Cooper J, Maubaret CG, Stephens JW, Abelak K, et al. Association of telomere length with type 2 diabetes, oxidative stress and UCP2 gene variation. Atherosclerosis. 2010; 209:42–50.

27. Schürks M, Prescott J, Dushkes R, De Vivo I, Rexrode KM. Telomere length and ischaemic stroke in women: a nested case-control study. Eur. J. Neurol. 2013; 20:1068–74.

28. Shen Q, Zhao X, Yu L, Zhang Z, Zhou D, Kan M, et al. Association of leukocyte telomere length with type 2 diabetes in mainland Chinese populations. J. Clin. Endocrinol. Metab. 2012; 97:1371–4.

29. Weischer M, Bojesen SE, Cawthon RM, Freiberg JJ, Tybjærg-Hansen A, Nordestgaard BG. Short telomere length, myocardial infarction, ischemic heart disease, and early death. Arterioscler. Thromb. Vasc. Biol. 2012; 32:822–9.

30. Willeit P, Willeit J, Brandstätter A, Ehrlenbach S, Mayr A, Gasperi A, et al. Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. Arterioscler. Thromb. Vasc. Biol. 2010; 30:1649–56.

31. Yang Z, Huang X, Jiang H, Zhang Y, Liu H, Qin C, et al. Short telomeres and prognosis of hypertension in a chinese population. Hypertension. 2009; 53:639–45.

32. You N-CY, Chen BH, Song Y, Lu X, Chen Y, Manson JE, et al. A prospective study of leukocyte telomere length and risk of type 2 diabetes in postmenopausal women. Diabetes. 2012; 61:2998–3004.

33. Zee RYL, Castonguay AJ, Barton NS, Germer S, Martin M. Mean leukocyte telomere length shortening and type 2 diabetes mellitus: a case-control study. Transl. Res. 2010; 155:166–9.

34. Zee RYL, Michaud SE, Germer S, Ridker PM. Association of shorter mean telomere length with risk of incident myocardial infarction: a prospective, nested case-control approach. Clin. Chim. Acta. 2009; 403:139–41.

35. Zee RYL, Castonguay AJ, Barton NS, Ridker PM. Relative leukocyte telomere length and risk of incident ischemic stroke in men: A prospective, nested case-control approach. Rejuvenation research. 2010; 13:411-14

36. Zhang W, Chen Y, Wang Y, Liu P, Zhang M, Zhang C, et al. Short telomere length in blood leucocytes contributes to the presence of atherothrombotic stroke and haemorrhagic stroke and risk of post-stroke death. Clin. Sci. (Lond). 2013; 125:27–36.

37. Zhao J, Zhu Y, Lin J, Matsuguchi T. Short Leukocyte Telomere Length Predicts Risk of Diabetes in American Indians: The Strong Heart Family Study. Diabetes. 2013:1–34.

38. Aubert G, Hills M, Lansdorp PM. Telomere length measurement-caveats and a critical assessment of the available technologies and tools. Mutat. Res. 2012; 730:59–67.

39. Diez Roux AV, Ranjit N, Jenny NS, Shea S, Cushman M, Fitzpatrick A, Seeman T. Race/ethnicity and telomere length in the Multi-Ethnic Study of Atherosclerosis. Aging Cell. 2009; 8:251–7.

40. Wells GA, Shea B, Connell DO, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta- analyses.

41. Whelan RS, Kaplinskiy V, Kitsis RN. Cell death in the pathogenesis of heart disease: mechanisms and significance. Annu. Rev. Physiol. 2010; 72:19–44.

42. Graham SH, Chen J. Programmed cell death in cerebral ischemia. J. Cereb. Blood Flow Metab. 2001; 21:99–109.

43. Lahera V, Goicoechea M, de Vinuesa SG, Miana M, de las Heras N, Cachofeiro V, et al. Endothelial dysfunction, oxidative stress and inflammation in atherosclerosis: beneficial effects of statins. Curr. Med. Chem. 2007; 14:243–8.

44. Cawthon RM, Smith KR, O'Brien E, Sivatchenko A, Kerber R. Association between telomere length in blood and mortality in people aged 60 years or older. Lancet. 2003; 361:393–5.

45. Stern MP. Diabetes and cardiovascular disease. The "common soil" hypothesis. Diabetes. 1995; 44:369–74.

46. Codd V, Nelson CP, Albrecht E, Mangino M, Deelen J, Buxton JL, et al. "Identification of seven locai affecting mean telomere length and their association with disease." Nautre. 2013; 45:422-427

2.7. Tables & Figures

Table 2.1. Characteristics of included studies assessing the association between LTL and stroke.

Source	Study Design	Follow-Up (years)	Events, n	Non-Events, n	Co-morbidity	Ethnicity	LTL assay	Inter-Assay CV* (%)	NOS Quality Score†		
									S	C	E/O
Ding et al., 2012	Case-Control	-	1309	1309	-	Asian	qPCR	1.3	4	2	3
Jiang et al., 2013	Case-Control	-	150	150‡	-	Asian	qPCR	6.7	4	2	3
Zee et al., 2010	Case-Control	-	259§	259§	-	Mixed	qPCR	<2.0	3	2	3
Zhang et al., 2013	Case-Control	-	503	1801	-	Asian	qPCR	6.4	3	2	3
Schurks et al., 2013	Nested Case-Control	-	504‖	504‖	-	Mixed	qPCR	22	2	2	3
Ding et al., 2012	Cohort	5.0	137	721	Previous stroke	Asian	qPCR	1.3	2	2	3
Fitzpatrick et al., 2007	Cohort	7.0	42	357	-	Mixed	Southern blot	1.5	2	2	2

Fyhrquist et al., 2011	Cohort	>4.0	43	1228	Hypertension, left ventricular hypertrophy	Mixed	Southern blot	3.7	2	2	3
Willeit et al., 2010	Cohort	4.4	46	754	-	Caucasian	qPCR	2.4	2	2	2
Yang et al., 2009	Cohort	5.0	NR	NR	Hypertensive patients	Asian	qPCR	6.8	3	2	2

NR = Not Reported

*CV= standard deviation / mean of replicates run at different time points.

† Newcastle Ottawa Scale: S = Selection (scored out of 4), C= Comparability (scored out of 2), E/O= Exposure/Outcome (scored out of 3)

‡ Controls are matched siblings

§ Males Only ‖Females only

Table 2.2 Characteristics of included studies assessing the association between LTL and MI.

Source	Study Design	Follow-Up (years)	Events, n	Non-Events, n	Co-morbidity	Ethnicity	LTL assay	Inter-Assay CV* (%)	NOS Quality Score†		
									S	C	E/O
Brouilette et al., 2003	Case-Control	-	203	180	-	Caucasian	Southern blot	3.3	1	2	3
Zee et al., 2009	Case-Control	-	337‡	337‡	-	Mixed	qPCR	5.0	3	2	3
Fitzpatrick et al., 2007	Cohort	7.0	36	352	-	Mixed	Southern blot	1.5	2	2	2
Fyhrquist et al., 2011	Cohort	>4.0	69	1202	Hypertension, left ventricular hypertrophy	Mixed	Southern blot	3.7	2	2	3
Weischer et al., 2012	Cohort	17§, 6‖	929	18355	-	Caucasian	qPCR	9.0	3	2	3
Willeit et al., 2012	Cohort	4.4	43	757	-	Caucasian	qPCR	2.4	2	2	2

*CV= standard deviation / mean of replicates run at different time points.

† Newcastle Ottawa Scale: S = Selection (scored out of 4), C= Comparability (scored out of 2), E/O=

Exposure/Outcome

‡ Males Only

§ Copenhagen City Heart Study ‖Copenhagen General Population Study

Table 2.3. Characteristics of included studies assessing the association between LTL and type 2 diabetes.

Source	Study Design	Follow-Up (years)	Events, n	Non-Events, n	Co-morbidity	Ethnicity	LTL assay	Inter-Assay CV* (%)	NOS Quality Score†		
									S	C	E/O
Olivieri et al., 2009	Case-Control	-	103	104	-	Caucasian	qPCR	6.0	3	2	2
Salpea et al., 2010	Case-Control	-	569	448	-	Mixed	qPCR	5.6	2	1	2
Shen et al., 2012	Case-Control	-	1936	2080	-	Asian	qPCR	2.0	4	2	2
You et al., 2012	Case-Control	-	1668‡	2361‡	-	Hispanic	qPCR	5.7	3	2	2
Zee et al., 2010	Case-Control	-	432	424	-	Mixed	qPCR	5.0	4	2	3
Hovatta et al., 2012	Cohort	8.5	130	172	Impaired glucose tolerance	Caucasian	qPCR	14	2	2	3
Zhao et al., 2013	Cohort	5.5	292	2036	-	Native American	qPCR	6.9	3	2	2

NR = Not Reported

*CV= standard deviation / mean of replicates run at different time points.

† Newcastle Ottawa Scale: S = Selection (scored out of 4), C= Comparability (scored out of 2), E/O= Exposure/Outcome (scored out of 3)

‡ Females Only

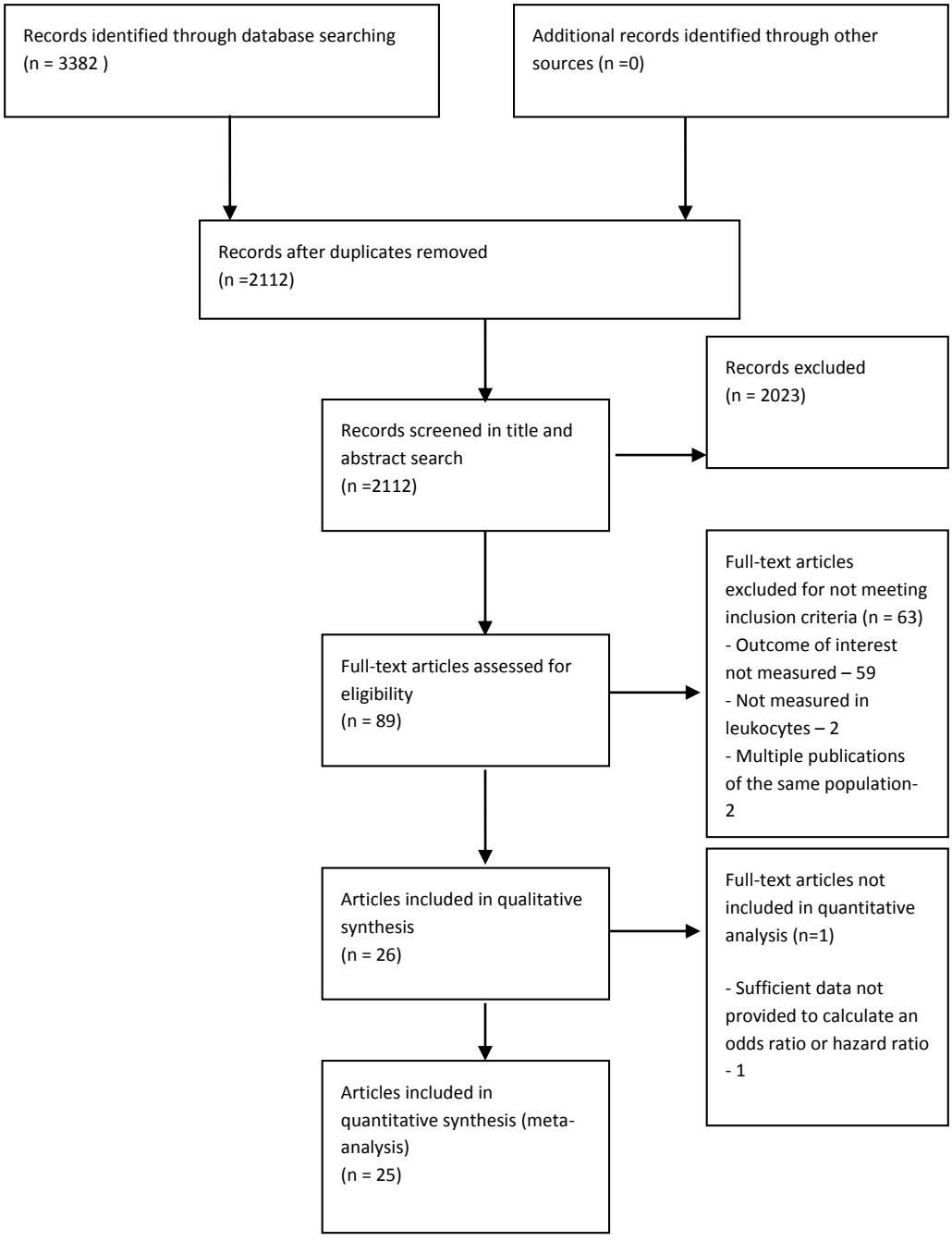


Figure 2.1. Flow diagram for the process of selecting eligible publications.

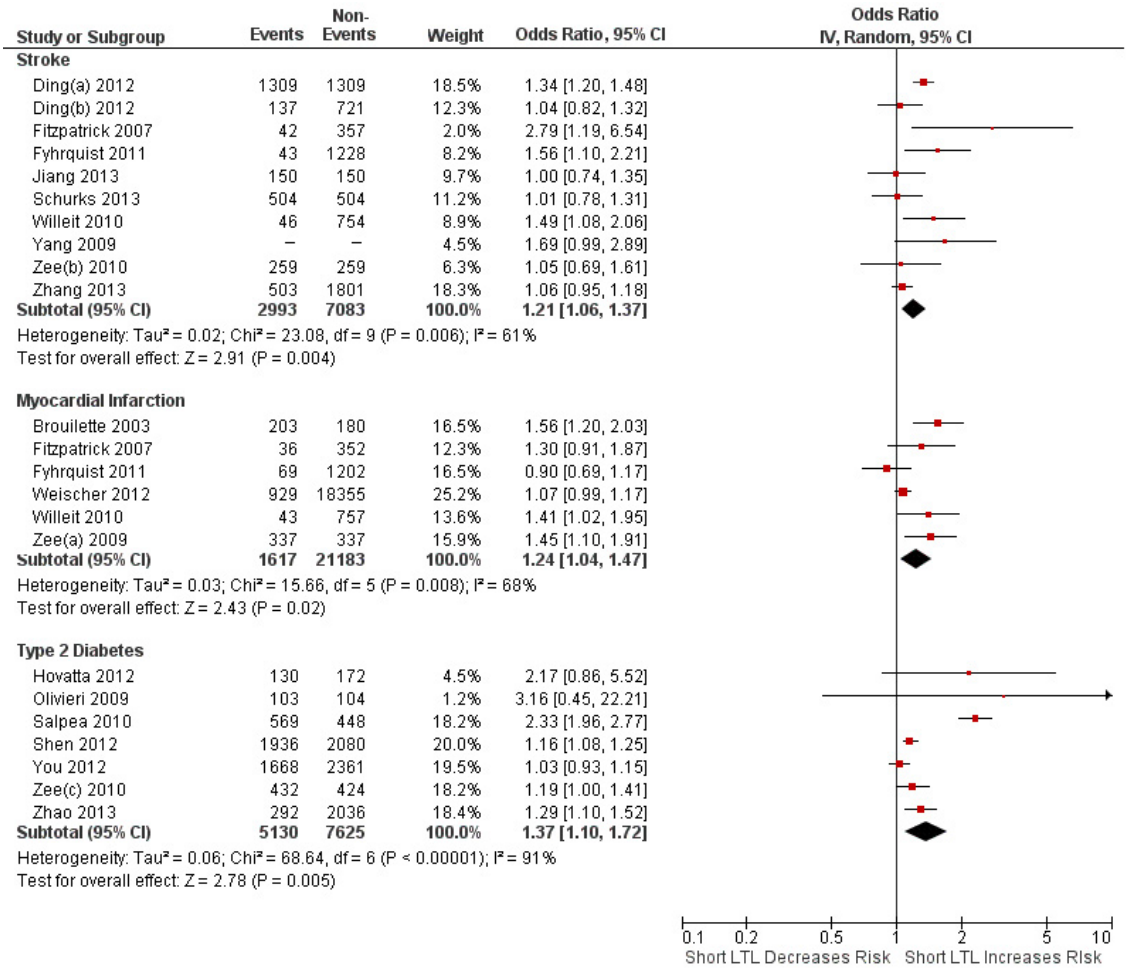


Figure 2.2 Forest plot of primary cardiometabolic outcomes. Results are presented for random effects models.

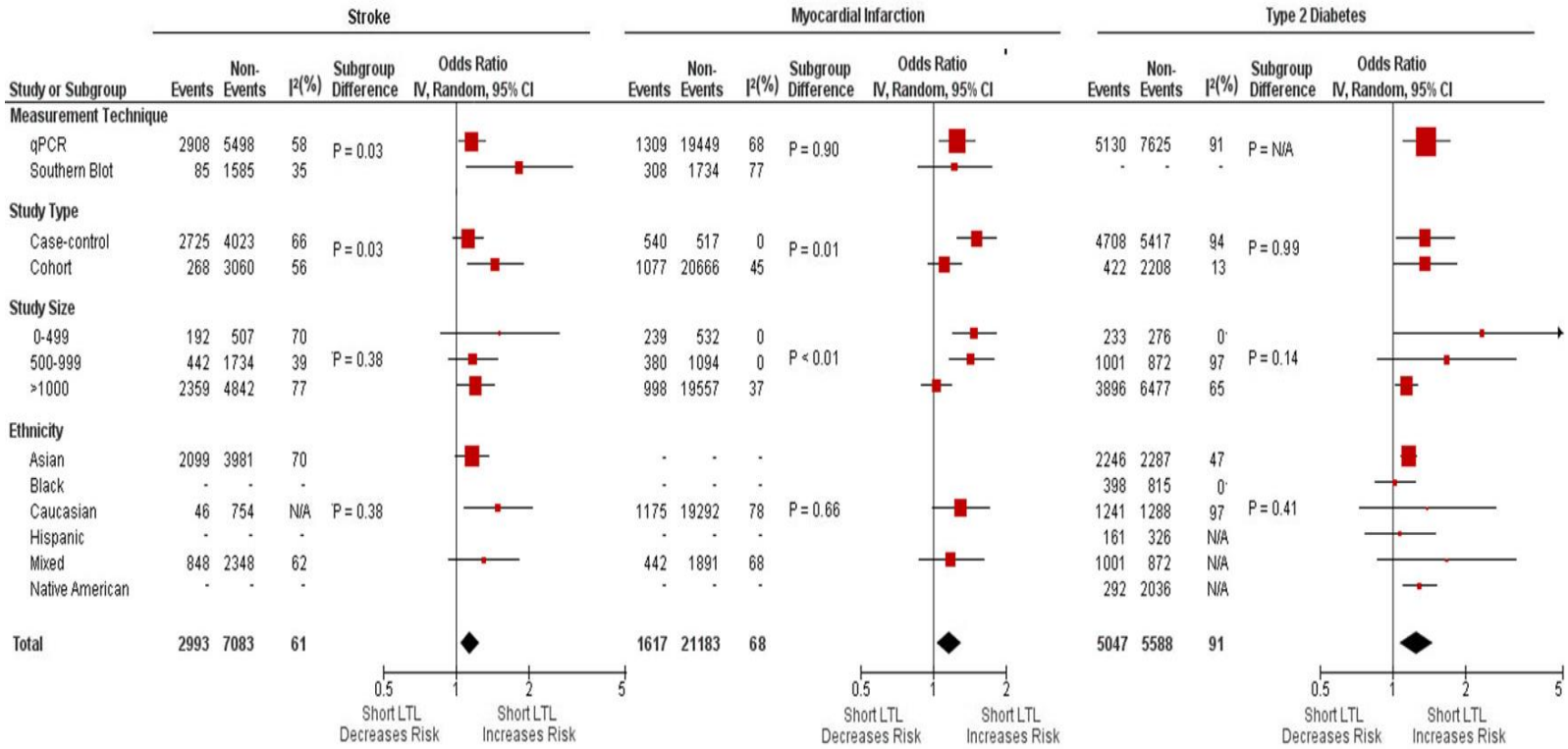


Figure 2.3. Summary of subgroup analyses for primary outcomes. Results are presented for random effects models.

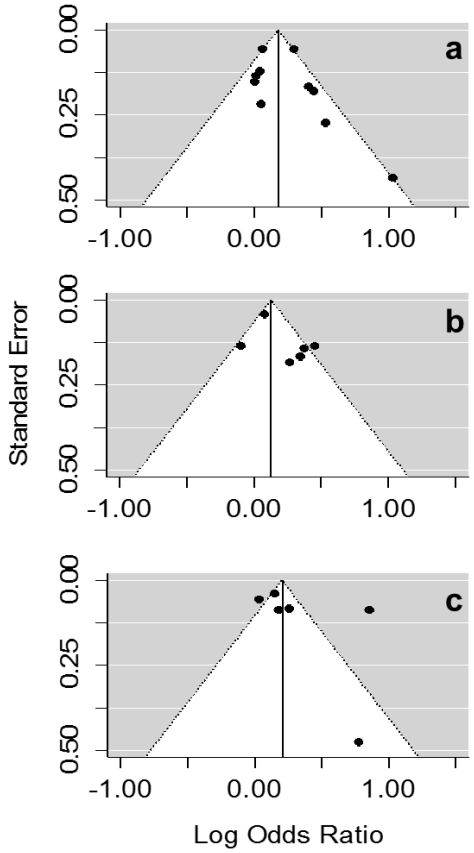


Figure 2.4. Funnel plots depicting level of publication bias within (a) stroke, (b) MI, and (c) T2D.

2.8. Supplementary Material

Supplemental Methods

Statistical Conversion Technique

ORs (and HRs) that used LTL as an ordinal variable and compared incidence of event in the shortest versus longest group were converted to per-SD decrease in LTL. To accomplish this we first obtained the beta value by taking the $\log_e$ of the OR. Assuming a normal distribution, we determined the standard deviation of the data. We then divided the beta by this standard deviation and exponentiated the result to obtain the OR per-SD decrease in LTL.

Supplementary Tables

Supplemental Table 2.1. Search Strategy for EMBASE (1980 - present) through OVID interface. Last conducted September 9th 2013

#	Searches	Results
1	telomere.mp. or exp telomere/ or exp telomere shortening/	20265
2	stroke.mp. or exp cerebrovascular accident/	262193
3	myocardial infarction.mp. or exp heart infarction/	283573
4	diabetes.mp. or exp diabetes mellitus/	638259
5	cardiovascular disease.mp. or exp cardiovascular disease/	2811015
6	exp cardiovascular mortality/ or exp mortality/ or mortality.mp.	840645
7	exp death/ or death.mp, or exp heart death/	835628
8	2 or 3 or 4 or 5 or 6 or 7	4293436
9	1 and 8	2522
10	Limit 9 to human	1999

Supplemental Table 2.2. Search Strategy for MEDLINE (1946 – present) through OVID interface. Last conducted September 9th 2013

#	Searches	Results
1	telomere.mp. or exp telomere/ or exp telomere shortening/	16968
2	exp stroke, lacunar/ or exp stroke/ or stroke.mp.	185795
3	diabetes.mp. or exp diabetes mellitus, type 2/ or exp diabetes	418147
4	cardiovascular disease.mp. or exp cardiovascular diseases/	1884627
5	cerebrovascular.mp. or exp cerebrovascular disorders/	317226
6	myocardial infarction.mp. or exp myocardial infarction/	188203
7	mortality.mp. or exp mortality/	626231
8	exp death/ or death.mp.	570732
9	2 or 3 or 3 or 4 or 5 or 6 or 7 or 8	3051388
10	1 and 9	1627
11	limit 10 to humans	1383

Supplemental Table 2.3. Characteristics of included studies assessing the association between LTL and cardiovascular related death.

Source	Study Design	Follow-Up (years)	Events, n	Non-Events, n	Co-morbidity	Ethnicity	LTL assay	Inter-Assay CV* (%)	NOS Quality Score†		
									S	C	E
Epel et al., 2009	Cohort	12	53	182	-	Caucasian	qPCR	NR	3	2	1
Fitzpatrick et al., 2011	Cohort	6.1	103	1031	-	Mixed	Southern Blot	1.5	4	2	3
Houben et al., 2011	Cohort	7.0	17‡	186‡	-	Caucasian	qPCR	NR	3	2	2
Martin-Ruiz et al., 2005	Cohort	3.0	249	977	-	Caucasian	qPCR	2.1	3	0	2
Njajou et al., 2009	Cohort	10	69	714	-	Mixed	qPCR	NR	3	0	3
Willeit et al., 2010	Cohort	4.4	45	755	-	Caucasian	qPCR	2.4	2	2	2

NR = Not Reported

*CV= standard deviation / mean of replicates run at different time points.

† Newcastle Ottawa Scale: S = Selection (scored out of 4), C= Comparability (scored out of 2), E/O= Exposure/Outcome (scored out of 3)

‡ Males Only

Supplemental Table 2.4. Characteristics of included studies assessing the association between LTL and the MACE composite outcome.

Source	Study Design	Follow-Up (years)	Events, n	Non-Events, n	Co-morbidity	Ethnicity	LTL assay	Inter-Assay CV* (%)	NOS Quality Score†		
									S	C	E
Farzaneh-Far et al., 2008	Cohort	4.4	118	662	Outpatients with stable CAD	Caucasian	qPCR	2.4	3	2	2
Fyhrquist et al., 2011	Cohort	>4.0	134	1137	Hypertension, left ventricular hypertrophy	Caucasian	Southern Blot	3.7	2	2	3
Willeit et al., 2010	Cohort	4.4	88	712	-	Caucasian	qPCR	2.4	2	2	2

NR = Not Reported

*CV= standard deviation / mean of replicates run at different time points.

† Newcastle Ottawa Scale: S = Selection (scored out of 4), C= Comparability (scored out of 2), E/O= Exposure/Outcome (scored out of 3)

Supplemental Table 2.5. Adjustments reported for included studies assessing stroke

Source	Reported Analysis Adjustment
Ding et al., 2012	Age, sex, BMI, hypertension, diabetes, hyperlipidemia, smoking status
Jiang et al., 2013	Age, sex, hypertension, recent social pressures, HDL
Zee et al., 2010	Age, smoking status, time of follow up, randomization treatment group, BMI, hypertension, diabetes, hyperlipidemia
Zhang et al., 2013	Age, gender, BMI, systolic/diastolic BP, fasting glucose, triacylglycerol, total cholesterol, HDL/LDL, smoking status, alcohol intake, diabetes, history of hyper tension, previous CHD, family history of stroke
Schurks et al., 2013	Age, smoking, postmenopausal status, post-menopausal hormone use, Elevated cholesterol, hypertension, diabetes, CHD, alcohol consumption, aspirin use, BMI, physical activity, total cholesterol/HDL ratio, HbA1c, healthy dietary score
Ding et al., 2012	Age, sex, BMI, hypertension, diabetes, hyperlipidemia, smoking status
Fitzpatrick et al., 2007	Age, sex, race
Fyhrquist et al., 2011	Age, sex
Willeit et al., 2010	Age, sex, previous stroke, hypertension, pack-years of smoking, ferritin, high-sensitivity C-reactive protein, lipoprotein(a), LDL, HDL, physical activity, diabetes mellitus, alcohol consumption
Yang et al., 2009	Age, gender, hypertension

Supplemental Table 2.6. Adjustments reported for included studies assessing myocardial infarction

Source	Reported Analysis Adjustment
Brouilette et al., 2003	Age, sex, smoking status
Zee et al., 2009	Age, smoking status, follow-up, treatment group, BMI, hypertension, diabetes, hyperlipidemia
Fitzpatrick et al., 2007	Age, sex, race
Fyhrquist et al., 2011	Age, sex
Weischer et al., 2012	Age, gender, study, cholesterol, triglycerides, high-density lipoprotein cholesterol, c-reactive protein, use of lipid lowering therapy, BMI, hypertension, diabetes, smoking, heavy alcohol intake, physical inactivity
Willeit et al., 2012	Age, sex, previous MI, hypertension, pack-years of smoking, ferritin, high-sensitivity C-reactive protein, lipoprotein(a), LDL, HDL, physical activity, diabetes mellitus, alcohol consumption

Supplemental Table 2.7. Adjustments reported for included studies assessing cardiovascular death

Source	Reported Analysis Adjustment
Epel et al., 2009	Age, Sex
Fitzpatrick et al., 2011	Age, sex, African-American race, hypertension, diabetes (ADA), smoking status, history of CAD, stroke, CHF, c-reactive protein, interleukin-6
Houben et al., 2011	Age, smoking status, alcohol use, body mass index, education, marital status, physical activity, chronic diseases
Martin-Ruiz et al., 2005	Age censored
Njajou et al., 2009	None
Willeit et al., 2010	Age, sex, hypertension, pack-years of smoking, ferritin, high-sensitivity C-reactive protein, lipoprotein(a), LDL, HDL, physical activity, diabetes mellitus, alcohol consumption

Supplemental Table 2.8. Adjustments reported for included studies assessing type 2 diabetes

Source	Reported Analysis Adjustment
Monikarage et al., 2012	Age, sex, waist circumference, adiponectin, telomere length, TBARS, insulin resistance
Olivieri et al., 2009	Age, sex, glucose, HbA1C waist-to hip ratio
Salpea et al., 2010	Age
Shen et al., 2012	Age, sex, BMI, smoking, and drinking
You et al., 2012	Age, ethnicity, date of blood collection, clinical center, BMI physical activity, hormone therapy, alcohol consumption, smoking
Zee et al., 2010	Age, smoking status, BMI, menopausal status, sex
Hovatta et al., 2012	Age, sex, randomization group
Zhao et al., 2013	Age, sex, age-squared, BMI, fasting glucose, and triglyceride level at baseline

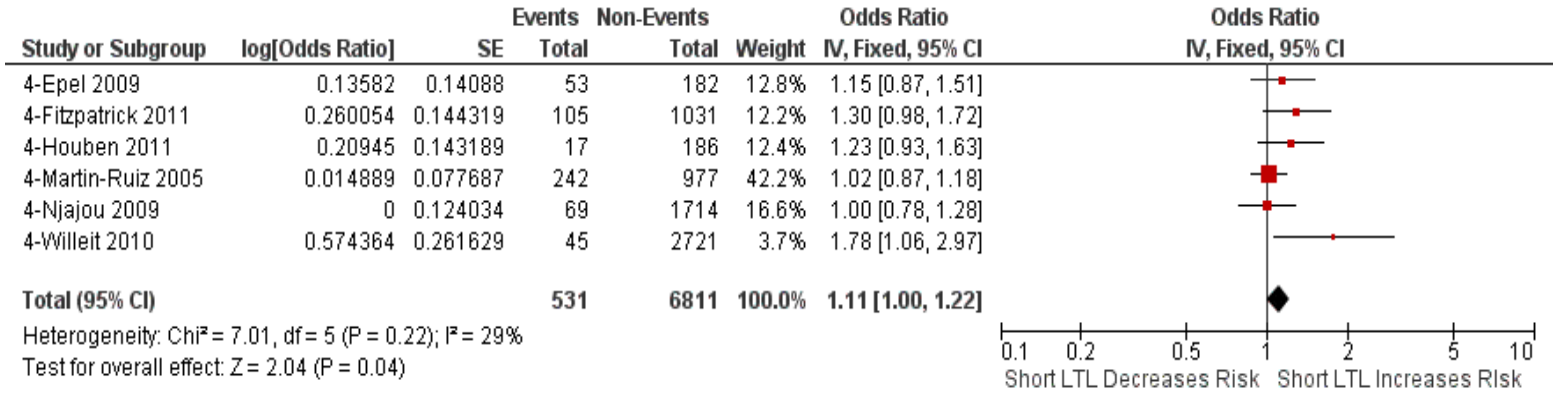
Supplemental Table 2.9. Adjustments reported for included studies assessing MACE composite outcome

Source	Reported Analysis Adjustment
Farzaneh-Far et al., 2008	Age, gender, ethnicity, LDL Cholesterol, HDL Cholesterol, Systolic Blood Pressure, Diastolic Blood Pressure, BMI, Stroke, Diabetes, CHF, Log CRP, LVEF, Diastolic dysfunction
Fyhrquist et al., 2011	Age, sex
Willeit et al., 2010	Age, sex, previous CVD, hypertension, pack-years of smoking, ferritin, high sensitivity C-reactive protein, lipoprotein(a), and low-/high-density lipoprotein cholesterol levels, physical activity, diabetes mellitus, alcohol consumption.

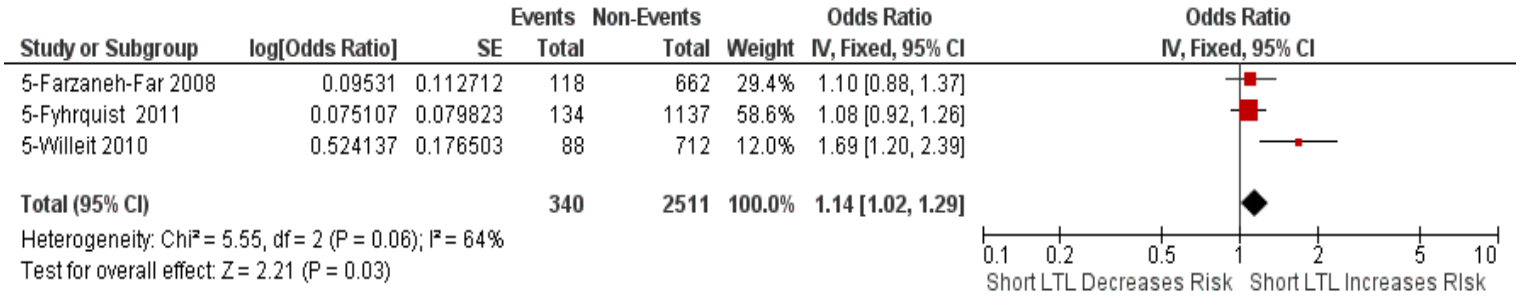
Supplemental Table 2.10. Sensitivity analysis removing studies with high inter-assay CV (>10%) and studies at a high or moderate risk of bias (as rated from NOS score).

	Stroke	MI	T2D	CVD related death	MACE
Original	1.21 [1.06, 1.37]	1.24 [1.04,1.47]	1.37 [1.10, 1.72]	1.11 [1.00,1.22]	1.14 [1.02,1.29]
Remove High CV	1.29 [1.19,1.40]	1.29 [1.13,1.47]	1.28 [1.21,1.36]	1.11 [0.97,1.26]	-
Remove Studies at Risk of Bias	-	-	1.15 [1.09, 1.21]	1.15 [1.04,1.26]	-

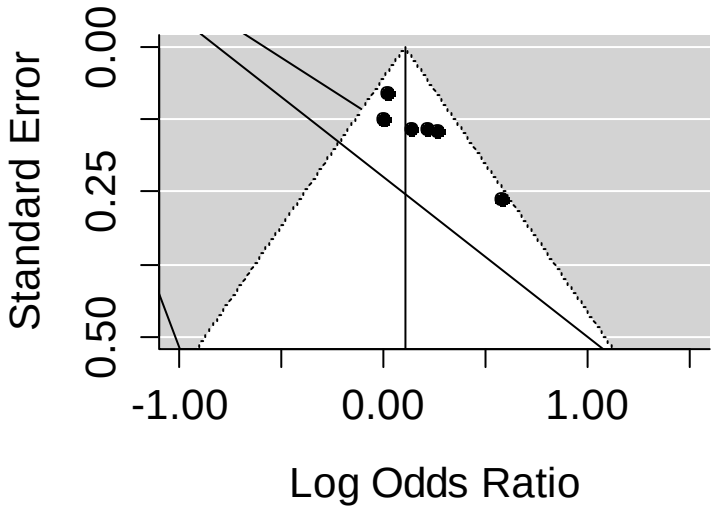
Supplementary Figures



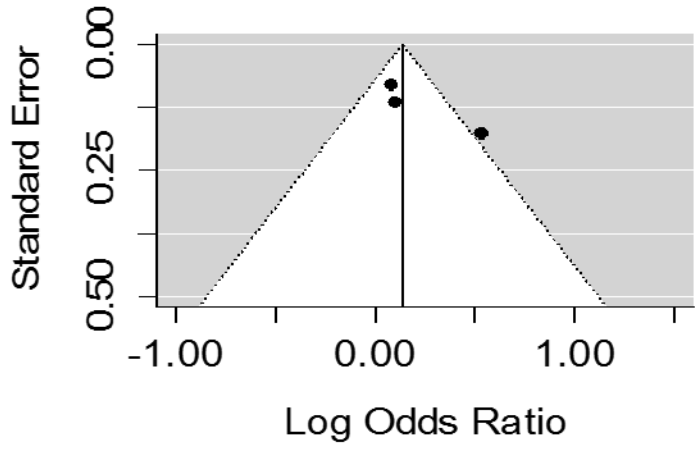
Supplemental Figure 2.1. Forest plot of studies assessing LTL and CVD death. Results are reported based on the fixed effect model.



Supplemental Figure 2.2. Forest plot of studies assessing LTL and a MACE composite outcome. Results are reported based on fixed effect model.



Supplemental Figure 2.3. Funnel plot of studies assessing LTL and CVD death



Supplemental Figure 2.4. Funnel plot of studies assessing LTL and a MACE composite outcome.

3. CHAPTER 3: A multi-ethnic evaluation of leukocyte telomere length as a biomarker for cardiometabolic outcomes: the INTERHEART and EpiDREAM study

3.1. Introduction

Telomeres are repetitive nucleotide sequences that cap the ends of chromosomes[1]. These gene poor regions help to stabilize the chromosome and prevent deleterious mutations from occurring. Telomeres naturally shorten as a result of cell division, but have also been shown to decrease in length due to oxidative stress[2,3] and chronic inflammation[4,5] caused by endogenous and environmental factors. Ultimately, apoptosis or cell cycle arrest (senescence) is triggered when telomeres reach a critically short length[6]. Cellular senescence is a key characteristic of many age-associated disorders and for this reason telomere length has been postulated to be a marker of biological ageing[7].

Senescence and tissue ageing are a central link between the pathologies of cardiometabolic outcomes such as cardiovascular disease (CVD) and type 2 diabetes (T2D)[8]. Marked by oxidative stress and chronic inflammation, these disease states are clearly related to age and have been increasingly studied in relation to telomere length. Indeed, many lines of evidence indicate telomere length does add predictive value (above chronological age and known risk factors) with respect to age-related diseases[10,11]. Thus, telomeres may not only be able to explain variability in the onset of cardiometabolic outcomes, but also reflect biological age.

To date, studies investigating the relationship between telomere length and individual cardiometabolic outcomes have been mostly case-control studies

with small sample sizes (n<1000) and variable telomere length measurement techniques[12–14]. Determining whether telomere length can be used to predict future cardiometabolic outcomes, as opposed to being merely a marker of them, is difficult because conflicting results are observed in studies that measure baseline telomere length in prospectively followed participants [15–17]. Importantly, the largest of association studies conducted have only looked at participants of a single ethnic group[15,17,18]. It is unknown whether reported effect estimates are applicable to a number of ethnic groups which experience a disproportionally greater burden of cardiometabolic events[19]. These groups would undoubtedly derive greater benefit from a predictor of such outcomes.

The aim of the current study is to investigate the association between telomere length and cardiometabolic outcomes after accounting for known cardiometabolic risk factors. Specifically, we will look at the association between telomere length and both myocardial infarction (MI) and T2D in INTERHEART, a large, international MI case-control study. Subsequently, we will assess the ability of telomere length to predict incident T2D, coronary artery disease (CAD) and major adverse cardiac events (MACE) in EpiDREAM, a second large, international prospective cohort study. Additional objectives include determining the relationship between telomere length and cardiometabolic risk factors, and verifying whether associations are consistent across various ethnic groups.

3.2. Methods

Study Population

The INTERHEART Study was a large, international, case-control study conducted between February 1999 and December 2002 to determine global risk factors for myocardial infarction (MI)[20]. Cases of any age were recruited from coronary care units (or equivalent wards) within 24 hours of presenting with clinical characteristics of acute MI. Controls, obtained from a community or hospital setting, had no history of CVD and were matched by age (± 5 years) and sex.

The Diabetes Reduction Assessment with Ramipril and Rosiglitazone Medication (DREAM) trial was a large, international, randomized controlled trial conducted between July 2001 and August 2003[21]. Individuals at high risk of T2D between the ages of 30 and 85 with impaired fasting glucose (6.1mmol/L – 7.0mmol/L) or impaired glucose tolerance (7.8 mmol/L - 11mmol/L) after 2 hour oral glucose load were randomized in a 2-by-2 factorial format to receive either Ramipril (15mg/day) or placebo and Rosiglitazone (8mg/day) or placebo. Participants were included in the epidemiological arm of the trial (EpiDREAM) if they either: 1) did not consent to be in the DREAM trial, but agreed to provide baseline information or to be prospectively followed, or 2) consented to be in the trial, but did not meet inclusion criteria. For the purposes of this study

EpiDREAM and DREAM will be referred to as EpiDREAM unless otherwise specified.

Outcome Assessment, Interim Contact, and Follow-up

Acute MI, the main outcome of interest in INTERHEART, was identified through patients presenting with clinical symptoms and new pathological Q waves, 1 mm ST elevation, left bundle branch block, persistent ST-T wave changes, or raised concentration (>2 times normal) of troponin. In EpiDREAM we defined two prospective cardiovascular outcomes: CAD and MACE. CAD comprised of MI, angina, or coronary artery bypass. MACE comprised of non-fatal stroke, non-fatal MI, or all-cause death. All events were adjudicated by a central committee for DREAM participants. For non-trial participants, events reported by subjects or relatives were confirmed by a recruiting center.

T2D, the main outcome of interest in EpiDREAM and was defined based on a fasting plasma glucose (FPG) ≥ 7.0mmol/L or a 2-hr post-load glucose ≥ 11.1mmol/L achieved during both an initial oral glucose tolerance test (OGTT) as well as a confirmatory test 3 months later. Diabetes status within INTERHEART was defined based on HbA1c ≥ 6.5% or self-reporting.

Participants of the DREAM trial were followed up every 3 months for incident T2D or CVD outcomes while non-trial participants that consented were contacted once in the second and fourth year after the initial screening visit. The

median (inter-quartile range) follow time up for all participants was 3.5 years (3.0-4.0 years).

Telomere Length Measurement

Telomere length was measured in leukocyte DNA extracted from peripheral blood samples. Leukocyte telomere length (LTL) is highly correlated with telomere length in various tissues[22] and is commonly measured within large epidemiological studies[15,23]. DNA was extracted from blood samples using the QIAsymphony (Qiagen) sample prep module for INTERHEART participants and the Autopure (Qiagen) sample prep module for EpiDREAM participants. DNA was eluted into TE buffer, stored at -80^{0}C, and thawed at 4^{0}C prior to use. A convenience sampling method was employed to select DNA samples for measurement as only samples with sufficient DNA (>10ng) were utilized. LTL was measured on the ViiA 7 real-time polymerase chain reaction system (Applied Biosystems) using a validated monochrome multiplex quantitative polymerase chain reaction method[24]. Briefly, this method controls for variations in input DNA by amplifying both telomere repeats (T) and a single-copy gene (S) within in each well. Consequently, a standardized ratio (T/S) can be calculated and used to compare telomere length across samples. The albumin gene was chosen as a single copy gene for this assay. A detailed description of the assay components and thermocycling parameters are provided in the supplementary materials. Samples were run in quadruplicate. A 3-fold dilution standard curve (300ng,

100ng, 33.3ng, 11.1ng, 3.70ng, 1.23ng) was run on each plate along with an interplate control to account for plate-to-plate variation. Overall, the standard curve efficiency for the telomere repeats and albumin was 91% and 89%, respectively. The assay was highly reproducible with average inter-assay and intra-assay coefficients of variation for the T/S ratio of 1.3% and 2.4%, respectively.

Statistical Analysis

Continuous variables are presented as mean (standard deviation) and dichotomous variables are presented as counts (percentage). For INTERHEART participants comparisons between continuous variables and categorical variables were made using Student's t-test and χ^2 test, respectively. For EpiDREAM participants trends across groups were identified using linear and logistic regression models in which glycemic status (normoglycemic, dysglycemic, or diabetic) was categorized as a continuous independent variable.

The distribution of measured telomere lengths was positively skewed and therefore T/S ratios were $\log_e$ transformed. These values were then multiplied by -1 for regression models in order to provide effect estimates based on decreasing telomere length. Linear regression was used to assess the association between LTL and common risk factors for cardiometabolic outcomes at baseline in INTERHEART controls and non-T2D EpiDREAM participants. Logistic regression was used to evaluate the association between LTL and MI in INTERHEART. The

area under the receiver operator curve (AUC) was used to assess model fit and the integrated discrimination index (IDI) was used to measure the improvement in discrimination after the addition of LTL to a fully adjusted model. Logistic regression was also used to evaluate the association between LTL and T2D, INTERHEART and EpiDREAM participants (combined) at baseline. INTERHEART participants with HbA1c<6% and normoglycemic EpiDREAM participants were considered T2D-controls. Cox-proportional hazard models were created to assess the predictive value of telomere length in EpiDREAM participants prospectively followed for cardiometabolic events. The proportional hazard assumption was confirmed through visually inspecting –log(log) graphs and verifying that Schoenfeld residuals were not time dependent ($p>0.05$). Subgroup analysis was carried out using the χ^2 test to assess heterogeneity between effect estimates. Population attributable risk (PAR) estimates were calculated using a method based on logistic regression which allowed for adjustment of confounders[20].

Minimally adjusted models accounted for age, sex, ethnicity, and variables found to be significantly associated with telomere length. For the cross-sectional analysis of T2D the minimally adjusted model also included terms for current/previous MI and DNA extraction technique. Fully adjusted models for CVD outcomes were additionally adjusted for INTERHEART risk factors: smoking status (current vs. former/never), diabetes, self-reported hypertension, ApoB:ApoA ratio, physical activity (any activity vs. sedentary lifestyle), weekly

alcohol consumption, waist to hip ratio, fruits and vegetables consumption (both vs. none), and general stress. Fully adjusted models pertaining to T2D were additionally adjusted for self-reported hypertension, smoking status (current vs. former/never), waist to hip ratio, and physical activity (any activity vs. sedentary lifestyle). Hazard estimates including DREAM participants included a variable to account for randomization group.

Two sided p-values <0.05 were considered significant unless otherwise stated. All statistical analyses were performed using R Statistical Package.

3.3. Results

Baseline characteristics of INTERHEART and EpiDREAM participants are presented in Table 3.1. LTL measurements were obtained for a total of 8293 participants from INTERHEART and 11026 from EpiDREAM. 78.3% (n=8635) of EpiDREAM participants with baseline LTL measurements were followed prospectively (Supplementary Table 3.1). INTERHEART cases had significant shorter LTL than controls ($p=3.37x10^{-21}$). They were also more likely to be diabetic, overweight, hypertensive, sedentary, current smokers, and have lower ApoA and higher ApoB levels than controls ($p<0.01$ for all). No linear trend in LTL was observed across EpiDREAM participants categorized as normoglycemic, dysglycemic, or diabetic at baseline (p-trend=0.81). Conversely, a linear increase in age, percent male sex, percent hypertensive, waist to hip ratio, and ApoB levels was observed with increasing glycemic status ($p<0.01$ for

all). A linear decrease in physical activity and ApoA levels was also observed with increasing glycemic status ($p<0.01$ for both).

Cardiometabolic Risk Factors and LTL

Age and ethnicity were factors significantly influencing LTL in controls from INTERHEART and non-diabetic participants from EpiDREAM (Supplementary Tables 3.2 and 3.3). After adjusting for sex and ethnicity, each year increase in age was associated with a similar decrease in LTL (INTERHEART: $\beta=0.003$, $SE=4.01x10^{-4}$, $p=1.3x10^{-16}$; EpiDREAM normoglycemic: $\beta=0.003$, $SE=0.001$, $p=9.2x10^{-8}$; EpiDREAM dysglycemic: $\beta=0.004$, $SE=7.09x10^{-4}$, $p=3.2x10^{-9}$). Membership of any ethnic group was associated with shortened LTL compared to Caucasians after accounting for age and sex differences within INTERHEART controls. Conversely, in EpiDREAM normoglycemic participants, the South Asian ($\beta=-0.093$, $SE=0.018$, $p=1.0x10^{-7}$) and Latin American ($\beta=-0.110$, $SE=0.017$, $p=4.4x10^{-11}$) ethnic groups were associated with an increased LTL in comparison to Caucasians. In dysglycemic EpiDREAM participants, the South Asian ethnic group was associated with decreased LTL ($\beta=0.147$, $SE=0.025$, $p=6.5x10^{-9}$), while the African group was associated with increased LTL ($\beta=-0.092$, $SE=0.029$, $p=0.001$). Lastly, increased LDL levels were associated with longer LTL in INTERHEART participants after accounting for age, sex, and ethnicity ($\beta=-0.014$, $SE=0.004$, $p=0.001$).

LTL and Cardiometabolic Outcomes

Within INTERHEART each unit decrease in LTL was associated with an increased risk of MI (OR=2.17, 95% CI=1.74-2.72) in the fully adjusted model. The risk of MI increased linearly (p-trend=$1.35x10^{-4}$) when LTL was categorized in tertiles and shorter tertiles were compared to the longest (middle vs. longest: OR=1.39, 95% CI=1.22-1.58; shortest vs. longest: OR= 1.77, 95% CI=1.55-2.02). The addition of LTL did not significantly improve the fully adjusted model (IDI=0.007; z=0.359, p=0.359). See Supplementary Figure 3.1 for receiver operator curves. The association between LTL and T2D was not significant on a per unit basis nor across any of the tertiles (Table 3.2).

Subgroup exploration of T2D status, sex, hypertensive status, smoking status, ethnicity, and age group did not significantly change effect estimates for MI in INTERHEART participants and T2D in EpiDREAM participants (Figures 3.1 and 3.2). Significant heterogeneity (p=0.02) was observed in the association between LTL and T2D in EpiDREAM participants who were active on a weekly basis (OR=0.88, 95% CI= 0.76-1.01) and those who were sedentary (OR= 1.24, 95% CI= 0.97-1.57). When subdivided by ethnic group, PARs for MI due to LTL ranged from 5.50% for South Asians to 22.0% for Arabs (Supplementary Table 3.4).

LTL and Incident Cardiometabolic Outcomes

The hazard of cardiometabolic events based on decreasing LTL in EpiDREAM participants is presented in Table 3.3. During follow-up 219

participants experienced a CAD event: MI (n=58), angina (n=76), and revascularization (n=85). The HR of CAD per unit decrease in LTL was 0.92 (95% CI= 0.66-1.29). No trend was observed across tertiles (p-trend=0.80). Furthermore, during follow-up 172 participants experienced a MACE event: MI (n=58), stroke (n=31), all-cause mortality (n=83). The HRs observed per unit decrease in LTL and across each tertile were not significant. The hazards for individual CVD outcomes are reported in Supplementary Table 3.5. Lastly, 800 participants developed T2D during follow-up. The HR per unit decrease in LTL was 0.96 (95% CI= 0.83-1.11). No trend was observed across tertiles (p-trend=0.97).

Estimates of the hazard between decreasing LTL and T2D remained consistent in subgroup exploration of diabetic status, physically active participants, hypertensive state, ethnicity, and age group (Figure 3.3). Effect estimates differed significantly (p=0.04) for participants who had never smoked (HR= 0.81, 95% CI= 0.67-0.99) and those who were current or former smokers (HR=1.11, 95% CI= 0.89-1.37).

Sensitivity Analysis

Hazard estimates for T2D per unit decrease in LTL did not significantly differ (p=0.07) between DREAM and EpiDREAM participants (Supplementary Figure 3.2).

3.4. Discussion

In this investigation of the relationship between telomere length and cardiometabolic outcomes in two large, international studies we report a significant association between decreased LTL and MI. An association between LTL and diabetes was not observed in either study population nor was LTL found to be a significant predictor of prospective cardiometabolic events. Our study provides robust results primarily because of our large sample size (n=19319). Due to design of each study it is possible to evaluate telomere length not only as a marker for cardiometabolic outcomes, but also as a predictor for future events. Utilizing two independent studies allows us to confirm associations with a single LTL assay, thereby reducing the risk of spurious results. Also, the multi-ethnic study population recruited from over 52 countries offers a unique opportunity to explore whether risk estimates are consistent across various populations.

The observed association with MI in INTERHEART provides support for LTL having a strong predictive capacity above chronological age, sex, and known risk factors. Our reported 2.25 times increase in risk of MI per 1 unit decrease in LTL is consistent with previous cross-sectional studies, all considerably smaller than the present study. For instance, an adjusted OR of 1.50 (95% CI= 1.10-1.47) per unit decrease in LTL was reported for 337 patients with MI and 337 controls from the Physicians Health Study[25]. Biologically, the association between LTL and MI is compelling as vascular tissue senescence is a key

initiator of atherosclerosis which ultimately leads to acute MI[26]. Indeed, shorter telomeres, a marker of senescence, are present in vascular tissue with atherosclerotic lesions[27]. Short LTL is also strongly associated with advanced atherogenesis[10] and high risk plaque morphology[28].

A notable finding was the consistency of effect estimates across six ethnic groups after adjusting for known risk factors for MI (p for heterogeneity=0.19). It is intriguing to speculate that unexplained differences in disease burden between ethnic groups may be reflected in varying strengths of association with telomere length. We have shown, conversely, that differential exposure to environmental or genetic risk factors associated with each ethnic group is more strongly reflected in baseline LTL differences than in strength of association with telomere length. It is also possible that individual ethnic groups have different rates of telomere attrition over time or different mean telomere length at birth, and as a result differences in baseline LTL may not directly translate into increased risk.

Interestingly, the association between baseline telomere length and specific ethnic groups was not consistent in both INTERHEART and EpiDREAM. For instance, the South Asian controls were associated with significantly shorter telomeres than Caucasian controls in INTERHEART (β=0.119, SE=0.016, $p=2.4x10^{-14}$). On the other hand, in EpiDREAM, normoglycemic South Asians were associated with longer telomeres than normoglycemic Caucasians (β=-0.093, SE=0.018, $p=1.0x10^{-7}$). These dissimilar observations are likely due to the

fact that all EpiDREAM participants were selected for being at high risk of T2D. As such, the distribution of various risk factors that influence telomere length may not be truly representative of differences commonly observed between ethnic groups.

Furthermore, our findings suggest that LTL is a poor predictor of future CVD events in individuals at high risk for diabetes (CAD: HR=0.92, 95% CI= 0.66-1.29). To date, the largest study assessing ischemic heart disease in two population-based cohorts (2038 events /19 838 participants) reported a very modest association with LTL (HR= 1.06, 95% 1.00-1.11 per 1000 bp decrease in LTL) over a 19 year follow-up period[15]. Our conflicting findings are most likely caused by a lack of power to detect significant associations due to too few events (n=219) over a relatively short follow-up time (3.5 years). Of note, we cannot exclude the possibility of confounding factors. Indeed, a causal association between LTL and CAD has been demonstrated previously through an association between single nucleotide polymorphisms (SNPs) related to decreased LTL and an increased risk of CAD[29]. Genetic variants such as SNPs are not influenced by environment exposures and therefore better represent the relationship between LTL and CAD.

The results of our study do not lend support for the use of telomere length as a marker for T2D or predictor for incident T2D. After adjusting for common T2D risk factors we were unable to find a significant association between LTL

and T2D. This is in contrast to a study including 1936 T2D cases and 2080 controls from mainland China which reported an OR of 1.52 (95% CI= 1.23-1.88) for T2D per unit decrease in LTL[18]. A possible explanation for this discrepancy is the extreme sampling used to obtained controls in their study. Unlike INTERHEART which used hospital-based controls free of CVD and EpiDREAM that selectively sought individuals at risk for diabetes, controls in their study were free of all psychological and physical disabilities, cancer, stroke, CVD, and any communicable disease. Thus, differences in participant telomere length within their study could reflect lesser biological aging in controls rather than accelerated aging in T2D cases. Nevertheless, our findings are in agreement with results from the Women's Health Initiative study (WHIS) in which no LTL-T2D association was found across four ethnic groups ($n_{Caucasian}$=2035, n_{Black}=1219, $n_{Hispanic}$=493, and n_{Asian}=308), after adjusting for known risk factors[16]. Moreover, SNPs associated with short LTL in WHIS participants were not associated with increased risk of T2D, indicating a lack of genetic support for a causal relationship[16].

There are some limitations to this study that warrant discussion. First, telomere length is reported as a relative T/S ratio instead of nucleotide bases and as such, risk cannot be estimated based on base differences. Moreover, ascertainment of diabetes status differed between INTERHEART and EpiDREAM. INTERHEART relied on HbA1c percentage, whereas EpiDREAM used OGTT. Both have been previously found to be moderately correlated in

their ability to assess glycemic control[30]. Lastly, despite our large sample size, power remains limited to detect interactions in EpiDREAM with respect to T2D. Significant heterogeneity was observed for physical activity status (and baseline T2D), and smoking status (incident T2D). While intriguing, these findings do not survive adjustment for multiple hypothesis testing.

3.5. Conclusion

In summary, this study provides evidence supporting telomere length as a universal marker of MI above known risk factors. The lack of an association with T2D suggests that all age-related diseases do not reflect the same degree of biological aging within the human body. Thus, LTL may serve as a better biomarker for some age-associated diseases than others. Population based studies with longer follow-up periods must be conducted in the future to clearly elucidate the relationship between telomere length for incident cardiometabolic outcomes.

3.6. References

1. Blackburn, E. H. Structure and function of telomeres. *Nature* **350,** 569–73 (1991).

2. Demissie, S. *et al.* Insulin resistance, oxidative stress, hypertension, and leukocyte telomere length in men from the Framingham Heart Study. *Aging Cell* **5,** 325–30 (2006).

3. Matthews, C. *et al.* Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. *Circ. Res.* **99,** 156–64 (2006).

4. Masi, S. *et al.* Inflammation and not cardiovascular risk factors is associated with short leukocyte telomere length in 13- to 16-year-old adolescents. *Arterioscler. Thromb. Vasc. Biol.* **32,** 2029–34 (2012).

5. Aikata, H. *et al.* Telomere reduction in human liver tissues with age and chronic inflammation. *Exp. Cell Res.* **256,** 578–82 (2000).

6. Allsopp, R. & Harley, C. Evidence for a critical telomere length in senescent human fibroblasts. *Exp. Cell Res.* (1995). at <http://www.sciencedirect.com/science/article/pii/S001448278571213X>

7. Mather, K. A., Jorm, A. F., Parslow, R. A. & Christensen, H. Is telomere length a biomarker of aging? A review. *J. Gerontol. A. Biol. Sci. Med. Sci.* **66,** 202–13 (2011).

8. Kannel, W. B. & Daniel, L. Diabetes and Cardiovascular Disease The Framingham. (2013).

9. Sanders, J. L. & Newman, A. B. Telomere Length in Epidemiology: A Biomarker of Aging, Age-Related Disease, Both, or Neither? *Epidemiol. Rev.* **35,** 112–131 (2013).

10. Willeit, P. *et al.* Cellular aging reflected by leukocyte telomere length predicts advanced atherosclerosis and cardiovascular disease risk. *Arterioscler. Thromb. Vasc. Biol.* **30,** 1649–56 (2010).

11. Fitzpatrick, A. L. *et al.* Leukocyte telomere length and cardiovascular disease in the cardiovascular health study. *Am. J. Epidemiol.* **165,** 14–21 (2007).

12. Brouilette, S., Singh, R. K., Thompson, J. R., Goodall, A. H. & Samani, N. J. White cell telomere length and risk of premature myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* **23,** 842–6 (2003).

13. Zee, R. Y. L., Castonguay, A. J., Barton, N. S., Germer, S. & Martin, M. Mean leukocyte telomere length shortening and type 2 diabetes mellitus: a case-control study. *Transl. Res.* **155,** 166–9 (2010).

14. Olivieri, F. *et al.* Leukocyte telomere shortening in elderly Type2DM patients with previous myocardial infarction. *Atherosclerosis* **206,** 588–93 (2009).

15. Weischer, M. *et al.* Short telomere length, myocardial infarction, ischemic heart disease, and early death. *Arterioscler. Thromb. Vasc. Biol.* **32,** 822–9 (2012).

16. You, N.-C. Y. *et al.* A prospective study of leukocyte telomere length and risk of type 2 diabetes in postmenopausal women. *Diabetes* **61,** 2998–3004 (2012).

17. Zhang, W. *et al.* Short telomere length in blood leucocytes contributes to the presence of atherothrombotic stroke and haemorrhagic stroke and risk of post-stroke death. *Clin. Sci. (Lond).* **125,** 27–36 (2013).

18. Shen, Q. *et al.* Association of leukocyte telomere length with type 2 diabetes in mainland Chinese populations. *J. Clin. Endocrinol. Metab.* **97,** 1371–4 (2012).

19. Anand, S. S. *et al.* Differences in risk factors, atherosclerosis, and cardiovascular disease between ethnic groups in Canada: the Study of Health Assessment and Risk in Ethnic groups (SHARE). *Lancet* **356,** 279–284 (2000).

20. Yusuf, S. *et al.* Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* **364,** 937–52 (2004).

21. Gerstein, H. C., Yusuf, S., Holman, R., Bosch, J. & Pogue, J. Rationale, design and recruitment characteristics of a large, simple international trial of diabetes prevention: the DREAM trial. *Diabetologia* **47,** 1519–27 (2004).

22. Daniali, L. *et al.* Telomeres shorten at equivalent rates in somatic tissues of adults. *Nat. Commun.* **4,** 1597 (2013).

23. Weischer, M. *et al.* Short telomere length, cancer survival, and cancer risk in 47102 individuals. *J. Natl. Cancer Inst.* **105,** 459–68 (2013).

24. Cawthon, R. M. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res.* **37,** e21 (2009).

25. Zee, R. Y. L., Michaud, S. E., Germer, S. & Ridker, P. M. Association of shorter mean telomere length with risk of incident myocardial infarction: a prospective, nested case-control approach. *Clin. Chim. Acta.* **403,** 139–41 (2009).

26. Minamino, T. & Komuro, I. Vascular cell senescence: contribution to atherosclerosis. *Circ. Res.* **100,** 15–26 (2007).

27. Nzietchueng, R. *et al.* Telomere length in vascular tissues from patients with atherosclerotic disease. *J. Nutr. Health Aging* **15,** 153–6 (2011).

28. Calvert, P. A. *et al.* Leukocyte telomere length is associated with high-risk plaques on virtual histology intravascular ultrasound and increased proinflammatory activity. *Arterioscler. Thromb. Vasc. Biol.* **31,** 2157–64 (2011).

29. Codd, V. *et al.* Identification of seven loci affecting mean telomere length and their association with disease. *Nat. Genet.* **45,** 422–7, 427e1–2 (2013).

3.7. Tables & Figures

Table 3.1. Baseline characteristics of INTERHEART and EpiDREAM participants.*

	INTERHEART			EpiDREAM			
	Control	Case	p-value	Nornmo-glycemic	IFG and/or IGT	T2D	p-trend
n	4321	3972	-	5579	3906	1541	-
LTL, T/S Ratio	0.98 (0.42)	0.92 (0.37)	$3.37x10^{-21}$	1.11 (0.96)	1.16 (1.28)	1.12 (0.88)	0.81
Age, y	55.2 (12.1)	56.3 (12.0)	$2.34x10^{-05}$	50.5 (11.1)	55.1 (11.1)	56.1 (10.8)	$6.90x10^{-104}$
Male Sex, %	898 (20.8)	833 (21.0)	0.85	1982 (35.5)	1593 (40.8)	711 (46.1)	$4.02x10^{-16}$
Smoker[#], %	2211 (52.3)	2595 (67.9)	$3.20x10^{-47}$	2586 (0.46)	1759 (0.45)	751 (0.48)	0.37
Diabetes, %	382 (8.86)	783 (20.1)	$1.51x10^{-47}$	0 (0)	0 (0)	1541 (100)	-
Hypertension, %	928 (21.5)	1502 (38.4)	$6.60x10^{-63}$	1512 (27.1)	1724 (44.1)	742 (48.2)	$1.19x10^{-78}$
Waist to Hip Ratio	0.92 (0.08)	0.95 (0.08)	$6.87x10^{-46}$	0.87 (0.09)	0.90 (0.09)	0.92 (0.09)	$3.05x10^{-95}$
Physically Active	767 (17.8)	551 (14.3)	$2.27x10^{-05}$	765 (13.7)	467 (12.0)	175 (11.4)	$3.00x10^{-3}$
ApoA, g/L	1.20 (0.27)	1.10 (0.23)	$3.01x10^{-71}$	1.42 (0.32)	1.39 (0.30)	1.40 (0.30)	$2.95x10^{-5}$
ApoB, g/L	0.96 (0.25)	1.02 (0.27)	$1.90x10^{-31}$	0.90 (0.22)	0.94 (0.22)	0.98 (0.24)	$3.54x10^{-33}$
Ethnicity, %							
European	868 (20.1)	931 (23.4)	-	2883 (51.7)	2091 (53.5)	806 (52.3)	-
South Asian	609 (14.1)	591 (14.9)	-	838 (15.0)	458 (11.7)	208 (13.0)	-
South East Asian	860 (19.9)	925 (23.3)	-	57 (1.02)	53 (1.36)	32 (2.08)	-
Arabic	804 (18.6)	527 (13.3)	-	-	-	-	-
Hispanic	715 (16.5)	712 (17.9)	-	883 (15.8)	726 (18.6)	199 (12.9)	-
African	465 (10.8)	286 (7.20)	-	378 (6.78)	300 (7.68)	148 (9.60)	-
Native	-	-	-	212 (3.80)	116 (2.97)	46 (2.99)	-
Other	-	-	-	328 (5.88)	162 (4.15)	102 (6.62)	-

IFG, impaired fasting glucose; IGT, impaired glucose tolerance; T2D, type 2 diabetes; ApoA, apolipoprotein A1; ApoB, apolipoprotein B;

*Data are presented as mean (SD) unless otherwise indicated.

[#] Current/former vs never smoker

Table 3.2. Association between decreased LTL and cardiometabolic outcomes in INTERHEART and EpiDREAM study participants.

Outcome	Study	Cases/Controls	Model	OR (95% CI)				
					Tertiles Groups of LTL			
				Per Unit Decrease in LTL	Long	Medium	Short	p-trend
MI	INTERHEART	3972/4321	1#	2.05(1.75-2.40)	1.00	1.31(1.17-1.47)	1.70(1.52-1.90)	1.90x10-4
			2#	2.17(1.74-2.72)	1.00	1.39(1.22-1.58)	1.77(1.55-2.02)	1.35x10-4
T2D	INTERHEART + EpiDREAM	3260/9794	1$	1.02(0.92-1.13)	1.00	0.92(0.83-1.02)	0.97 (0.88-1.08)	0.63
			2$	0.99(0.89-1.11)	1.00	0.92(0.83-1.03)	0.98 (0.88-1.09)	0.80

MI, myocardial infarction; T2D, type 2 diabetes;

1#: adjusted for age, sex, and ethnicity

2#: Additionally smoking status, diabetes, hypertension, apoB:apoA ratio, physical activity, weekly alcohol consumption, waist to hip ratio, fruits and vegetables consumption, general stress, and low density lipoprotein

1$: adjusted for age, sex, ethnicity, study, and MI

2$: Additionally adjusted for smoking status, hypertension, physical activity, and waist to hip ratio

Table 3.3. Hazard of cardiometabolic outcomes based on decreased LTL in EpiDREAM study participants.

Outcome	Study (subset)	Events/ Total Participants	Model	HR (95% CI)				p-trend
					Tertile Groups of LTL			
				Per Unit Decrease in LTL	Long	Medium	Short	
CAD	EpiDREAM	160/8795	1	0.93(0.67-1.29)	1.00	0.89(0.59-1.34)	1.03(0.71-1.50)	0.83
		150/8409	2[S]	0.92(0.66-1.29)	1.00	0.85(0.56-1.30)	1.04(0.71-1.52)	0.80
MACE	EpiDREAM	167/8795	1	0.89(0.66-1.21)	1.00	0.93(0.64-1.35)	0.74(0.51-1.08)	0.70
		158/8409	2[S]	0.91(0.66-1.23)	1.00	0.89(0.61-1.31)	0.76(0.52-1.11)	0.70
T2D	EpiDREAM (non-T2D only)	800/7780	1	0.96(0.83-1.11)	1.00	0.95(0.80-1.13)	1.00(0.84-1.19)	0.97
		798/7748	2[#]	0.96(0.83-1.11)	1.00	0.96(0.81-1.15)	1.00(0.84-1.20)	0.95

CAD, coronary artery disease: myocardial infarction, angina, revascularization; MACE, major adverse cardiac event: myocardial infarction, stroke, all-cause mortality, T2D, type 2 diabetes;

Model 1: adjusted for age, sex, ethnicity and randomization group

Model 2[$]: additionally adjusted for hypertension, waist to hip ratio, physical activity, smoking status, diabetes status and apoB:apoA ratio

Model 2[#]: additionally adjusted for hypertension, waist to hip ratio, physical activity, smoking status

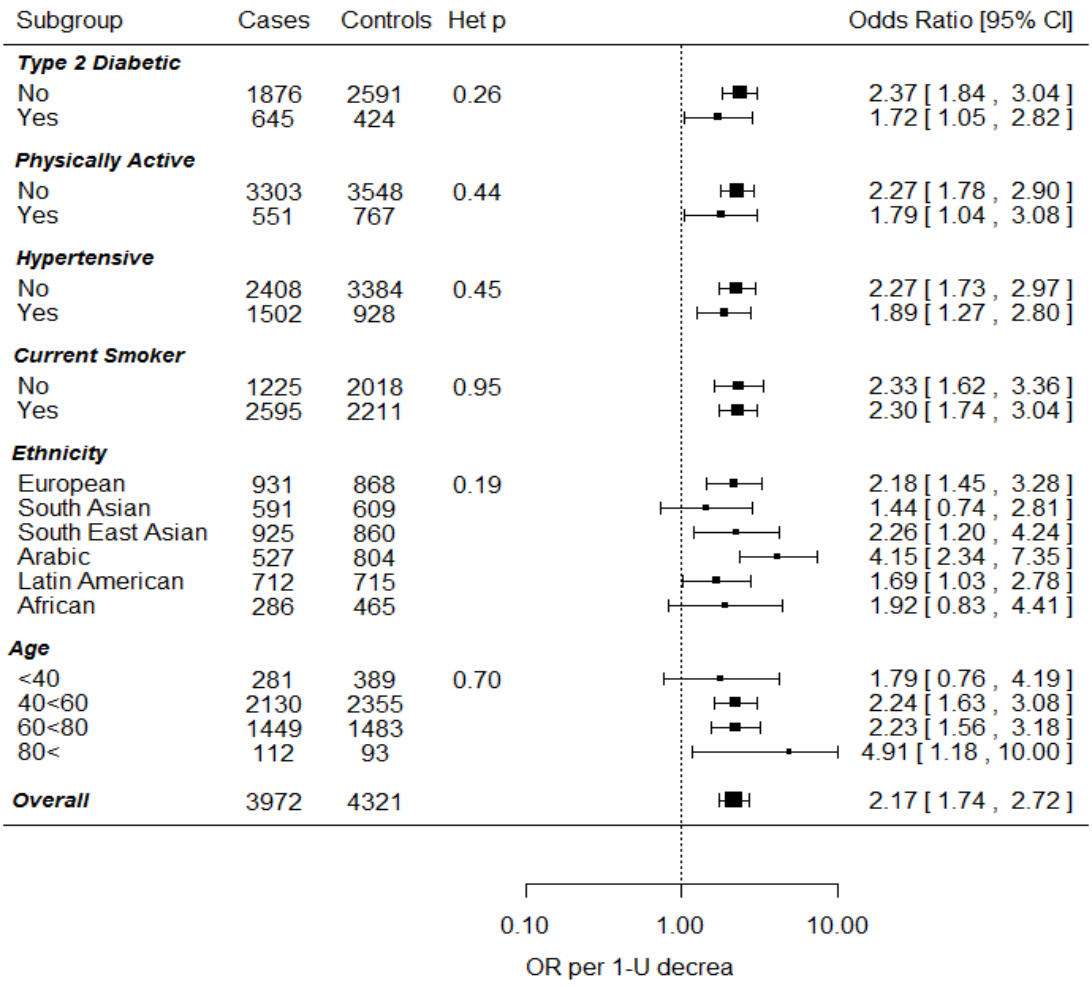


Figure 3.1. Subgroup analysis of the association of decreased LTL with MI in INTERHEART participants at baseline. Analyses were adjusted for age, sex, smoking status, diabetes status, hypertension status, ApoB:ApoA ratio, physical activity, alcohol consumption, waist to hip ratio, fruits and vegetable intake, home stress, LDL, and self-reported ethnicity (when appropriate). Het p, χ^2p-value for heterogeneity;

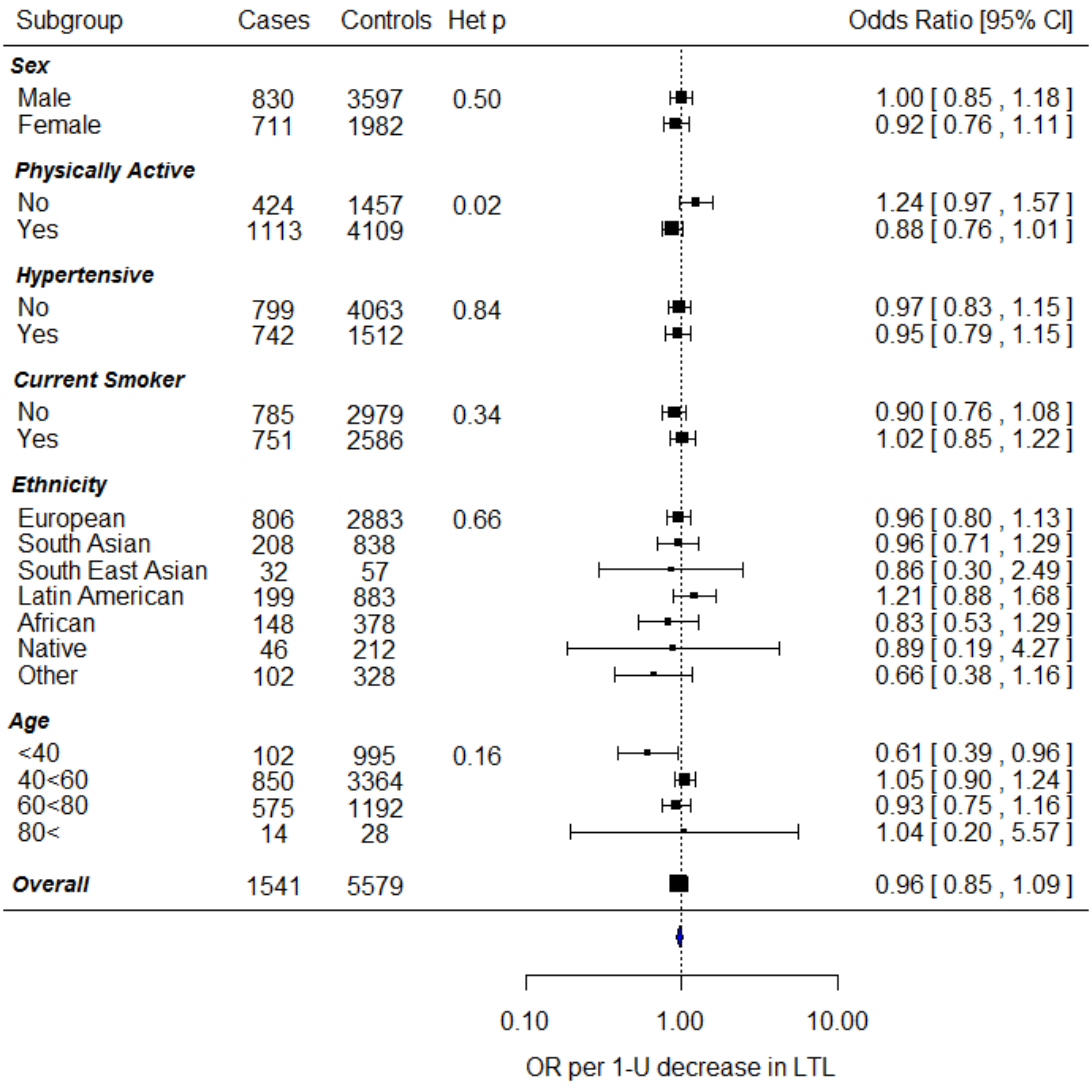


Figure 3.2. Subgroup analysis of the association of decreased LTL with T2D in EpiDREAM participants at baseline. Analyses were adjusted for age, sex, hypertension status, waist to hip ratio, physical activity, smoking status, and self-reported ethnicity (when appropriate). Normoglycemic participants used as controls. Het p, χ^2p-value for heterogeneity;

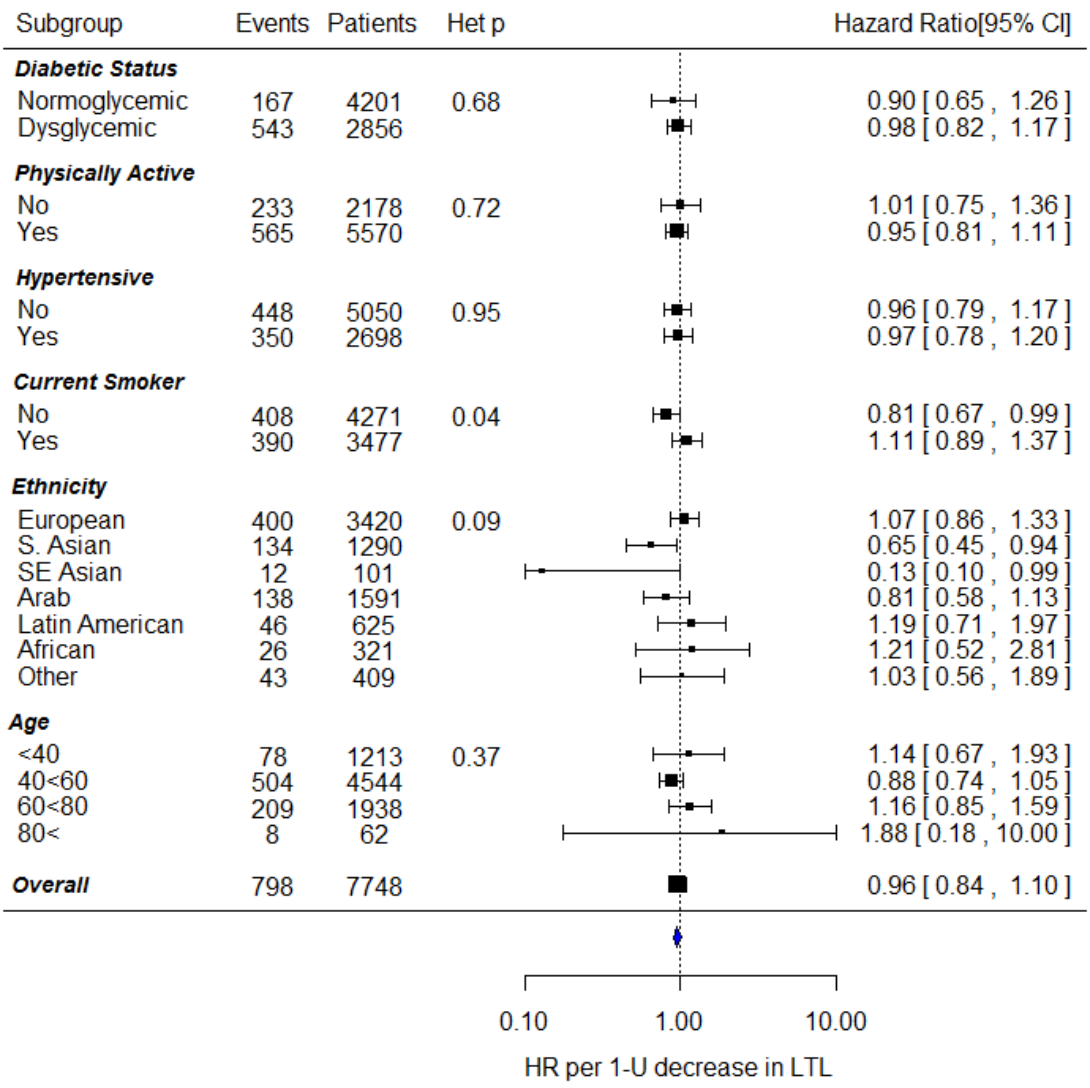


Figure 3.3. Subgroup analysis of the association of decreased LTL with prospective T2D in EpiDREAM participants. Analyses were adjusted for age, sex, randomization group, hypertension status, waist to hip ratio, physical activity, smoking status, and self-reported ethnicity (when appropriate). Het p, χ^2p-value for heterogeneity;

3.8. Supplementary Materials

Supplementary Methods

The reaction mixture (4uL per well) for the monochrome multiplex qPCR comprised of:

- 1X QuantiFast SYBR Green Master Mix (Qiagen)
- 300 nmol/L forward telomere primer (Sigma-Aldrich)
    - (5'ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-'3)
- 300 nmol/L reverse telomere primer (Sigma-Aldrich)
    - (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-'3)
- 350 nmol/L forward albumin primer (Sigma-Aldrich)
    - (5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-'3)
- 350 nmol/L reverse albumin primer (Sigma-Aldrich)
    - (5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-'3)

1uL of DNA sample was dispensed into 4uL of reaction mix using the Bravo Automated Liquid Handling System (Agilent Technologies).

The thermal cycling profile was set as follows on the ViiA7 system (Applied Biosystems):

- Stage 1: 15 min at 95°C;
- Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C;
- Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 73°C with signal acquisition,

  10 s at 84°C, 15 s at 87°C with signal acquisition;

Supplementary Table 3.1. Baseline characteristics EpiDREAM participants followed prospectively*

	EpiDREAM			
	Nornmo-glycemic	IFG and/or IGT	T2D	p-trend
n	4214	3566	1140	-
LTL, T/S Ratio	1.12 (0.92)	1.16 (1.32)	1.11 (0.85)	0.24
Age, y	49.40 (11.1)	54.80 (11.1)	55.30 (11.1)	$6.18x10^{-97}$
Male Sex, %	1502 (35.6)	1429 (40.1)	519 (45.5)	$1.19x10^{-10}$
Smoker[#], %	1894 (44.9)	1597 (44.8)	544 (47.7)	0.20
Diabetes, %	0(0)	0(0)	1140 (100)	-
Hypertension, %	1128 (26.8)	1584 (44.4)	529 (46.4)	$4.37x10^{-59}$
Waist to Hip Ratio	0.87 (0.09)	0.90 (0.09)	0.92 (0.08)	$2.65x10^{-71}$
Physically Active	516 (12.3)	401 (11.3)	110 (9.65)	0.01
ApoA, g/L	1.38 (0.30)	1.38 (0.29)	1.36 (0.28)	0.04
ApoB, g/L	0.88 (0.22)	0.94 (0.22)	0.97 (0.24)	$5.37x10^{-37}$
Ethnicity, %				
European	1644 (39.0)	1786 (50.1)	441 (38.7)	-
South Asian	833 (19.8)	457 (12.8)	206 (18.1)	-
South East Asian	51 (1.21)	50 (1.40)	31 (2.72)	-
Arabic	-	-	-	-
Hispanic	873 (20.7)	722 (20.2)	196 (17.2)	-
African	338 (8.02)	290 (8.13)	142 (12.5)	-
Native	205 (4.86)	116 (3.25)	42 (3.68)	-
Other	270 (6.41)	145 (4.07)	82 (7.19)	-

IFG, impaired fasting glucose; IGT, impaired glucose tolerance; T2D, type 2 diabetes; ApoA, apolipoprotein A1; ApoB, apolipoprotein B;

*Data are presented as mean (SD) unless otherwise indicated.

[#]Current/former vs never smoker

Supplementary Table 3.2. Risk factors associated with decreasing LTL in INTERHEART controls at baseline*

	INTERHEART Controls (n=4321)		
Risk Factor	β	SE	p-value
Age	0.003	$4.01x10^{-4}$	$1.3x10^{-16}$**
Male Sex	-0.012	0.012	0.303
Smoking (vs. Never)	-	-	-
Former	-0.007	0.012	0.587
Current	0.011	0.011	0.336
Diabetes	-0.002	0.015	0.903
Hypertension	0.004	0.011	0.724
Waist to Hip Ratio	-0.001	0.062	0.984
Fruits and Vegetables (vs. None)	-	-	-
Either	-0.014	0.013	0.283
Both	-0.021	0.013	0.127
Physically Active	0.025	0.012	0.041
Weekly Alcohol Intake	0.025	0.012	0.041
HDL	-0.036	0.013	0.006
LDL	-0.014	0.004	0.001**
Family History of MI	0.018	0.014	0.195
ApoA	-0.035	0.018	0.050
ApoB	-0.035	0.018	0.055
Work/Home Stress vs. None	-0.004	0.009	0.635
HbA1c	0.009	0.006	0.154
Ethnicity (vs. Caucasian)	-	-	-
South Asian	0.119	0.016	$2.4x10^{-14}$**
South East Asian	0.308	0.015	$2.1x10^{-93}$**
Arabic	0.118	0.015	$2.0x10^{-15}$**
Hispanic	0.081	0.015	$5.1x10^{-08}$**
African	0.103	0.017	$2.1x10^{-09}$**

HDL, High density lipoprotein; LDL, low density lipoprotein; ApoA, apolipoprotein A1; ApoB, apolipoprotein B; HbA1C, glycated hemoglobin;

* Analyses were adjusted for age, sex, and self-reported ethnicity (when appropriate)

**Significant at bonferroni corrected p-value < 0.05/23 = 0.002

Supplementary Table 3.3. Risk factors associated with decreasing LTL in EpiDREAM participants at baseline*

	Normoglycemic (n= 5579)			IFG and/or IGT (n=3906)		
Risk Factor	β	SE	p-value	β	SE	p-value
Age	0.003	0.001	$9.2x10^{-08}$**	0.004	$7.09x10^{-4}$	$3.0x10^{-09}$**
Male Sex	0.019	0.012	0.111	-0.017	0.015	0.269
Smoker (vs. Never)	-	-	-	-	-	-
Former	0.010	0.017	0.538	0.018	0.024	0.456
Current	0.025	0.014	0.067	-0.027	0.018	0.120
2 hour Plasma Glucose	-0.003	0.002	0.044	-0.003	0.004	0.502
Fasting Plasma Glucose	-0.011	0.004	0.006	0.009	0.012	0.439
Hypertension	-0.019	0.013	0.148	-0.010	0.016	0.515
Waist to Hip Ratio	0.086	0.075	0.251	0.048	0.101	0.634
Physically Active	0.013	0.020	0.530	-0.005	0.027	0.842
ApoA	-0.020	0.021	0.348	0.023	0.028	0.418
ApoB	0.007	0.026	0.774	-0.022	0.034	0.516
Ethnicity (vs. Caucasian)	-	-	-	-	-	-
South Asian	-0.093	0.018	$1.0x10^{-07}$**	0.147	0.025	$6.5x10^{-09}$**
South East Asian	-0.038	0.051	0.457	0.032	0.065	0.620
Hispanic	-0.110	0.017	$4.4x10^{-11}$**	0.003	0.020	0.891
African	-0.041	0.022	0.067	-0.092	0.029	0.001**
Native	0.237	0.031	0.237	-0.044	0.045	0.323
Other	0.851	0.024	0.851	-0.035	0.038	0.354

IGT, impaired glucose tolerance; IFG, impaired fasting glucose; ApoA, apolipoprotein A1; ApoB, apolipoprotein B; S, South; SE, South East;

* Analyses were adjusted for age, sex, and self-reported ethnicity (when appropriate)

**Significant at bonferroni corrected p-value < 0.05/19 = 0.003

Supplementary Table 3.4. Population attributable risk (PAR) for each ethnic group in INTERHEART.

Ethnicity	PAR due to 1-unit decrease in LTL (95% CI)*
European	16.6 (10.2-22.7)
South Asian	5.50 (1.08-9.75)
South East Asian	12.6 (3.41-21.3)
Arabic	22.0 (8.11-35.2)
Latin American	12.0 (3.00-21.0)
African	6.05 (2.03-11.4)

*PAR estimates adjusted for age, sex, smoking status, diabetes, hypertension, apoB:apoA ratio, physical activity, weekly alcohol consumption, waist to hip ratio, fruits and vegetables consumption, general stress, and low density lipoprotei

Supplementary Table 3.5. Hazard of composite and individual CVD endpoints based on decreased LTL in EpiDREAM study participants.

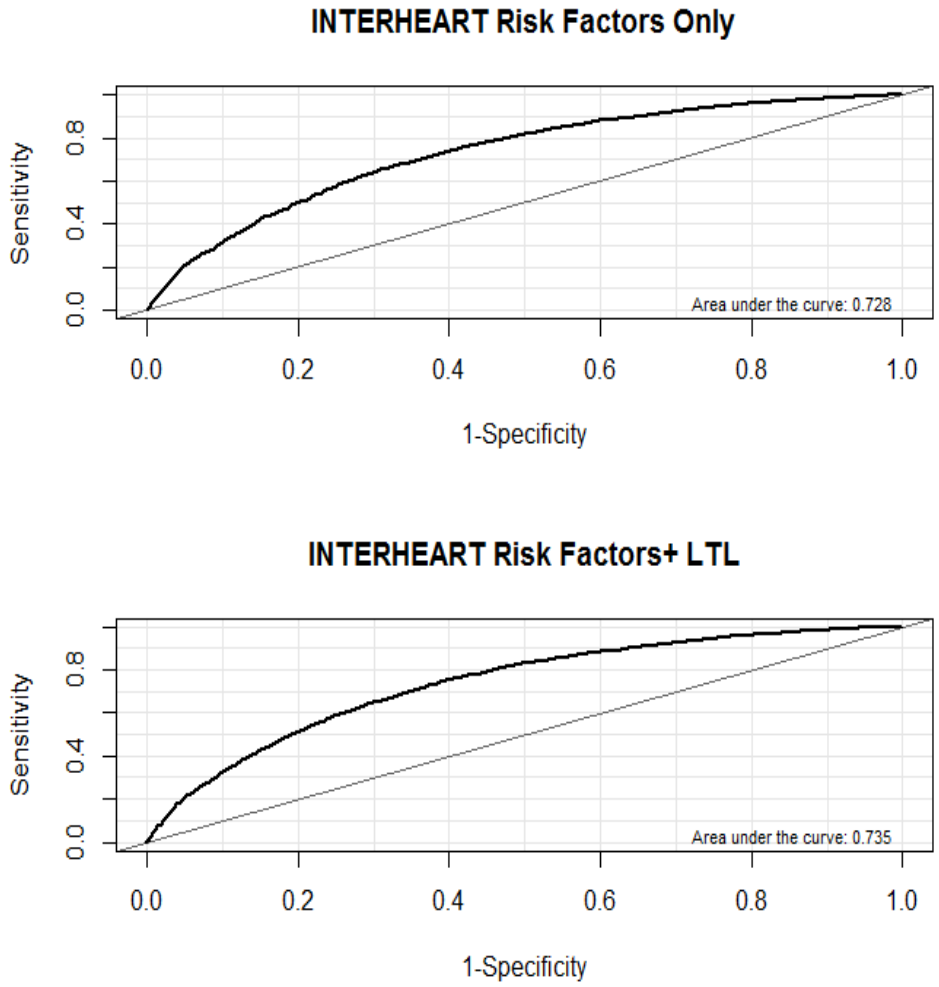
			HR (95% CI)				
				Tertile Groups of LTL			
Outcome	Events/ Total Participants	Model	Per Unit Decrease in LTL	Long	Medium	Short	p-trend
CAD	160/8273	1	0.93(0.67-1.29)	1.00	0.93(0.62-1.40)	1.03(0.71-1.50)	0.85
	150/7916	2	0.92(0.66-1.29)	1.00	0.90(0.59-1.36)	1.04(0.71-1.52)	0.82
MACE	167/8273	1	0.89(0.66-1.21)	1.00	0.94(0.65-1.37)	0.75(0.51-1.09)	0.76
	158/7916	2	0.91(0.66-1.23)	1.00	0.91(0.62-1.33)	0.77(0.52-1.13)	0.76
MI	58/8273	1	0.93(0.56-1.55)	1.00	1.13(0.59-2.17)	0.91(0.48-1.72)	0.70
	53/7916	2	0.91(0.54-1.53)	1.00	0.97(0.49-1.91)	0.89(0.46-1.71)	0.70
Stroke	31/8273	1	1.00(0.46-2.19)	1.00	1.96(0.78-4.92)	1.11(0.42-2.94)	0.15
	30/7916	2	1.01(0.46-2.23)	1.00	1.82(0.72-4.64)	1.15(0.43-3.06)	0.15
Angina	76/8273	1	0.86(0.54-1.37)	1.00	0.98(0.56-1.71)	0.88(0.5-1.53)	0.94
	73/7916	2	0.85(0.53-1.36)	1.00	1.00(0.56-1.77)	0.90(0.51-1.58)	0.94
Revascularization	85/8273	1	0.86(0.56-1.32)	1.00	1.08(0.63-1.87)	0.97(0.58-1.65)	0.78
	80/7916	2	0.86(0.55-1.33)	1.00	1.04(0.59-1.82)	0.98(0.57-1.69)	0.78
All Cause Death	83/8273	1	0.86(0.56-1.31)	1.00	0.59(0.34-1.03)	0.64(0.38-1.07)	0.06
	80/7916	2	0.89(0.58-1.36)	1.00	0.62(0.35-1.07)	0.68(0.40-1.15)	0.06

CAD, coronary artery disease: myocardial infarction, angina, revascularization; MACE, major adverse cardiac event: myocardial infarction, stroke, all-cause mortality, T2D, type 2 diabetes;

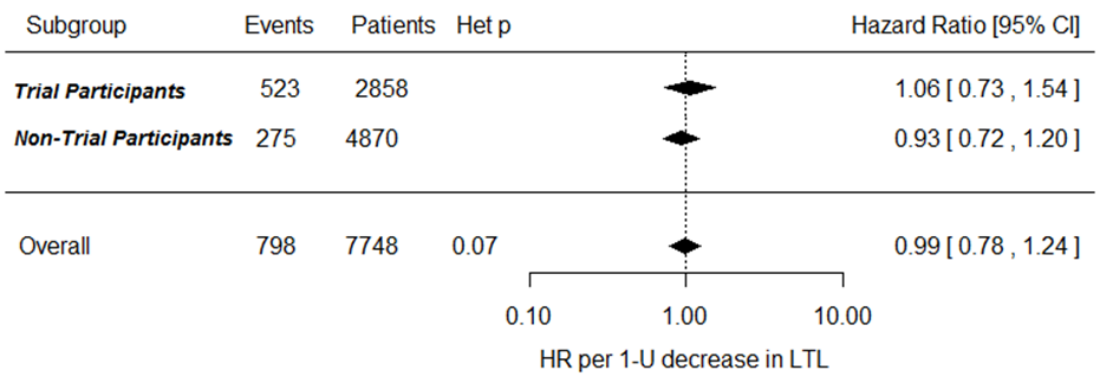
Model 1: adjusted for age, sex, ethnicity and randomization group

Model 2[$]: additionally adjusted for hypertension, waist to hip ratio, physical activity, smoking status, diabetes status and apoB:apoA ratio

Model 2[#]: additionally adjusted for hypertension, waist to hip ratio, physical activity, smoking status



Supplementary Figure 3.1a) Receiver operator curve (ROC) for INTERHEART risk factors only. This model has an AUC of 0.728 (95% CI= 0.722-0.734). 3.1b) ROC for INTERHEART risk factors and LTL. This full model includes the model in 1a) in addition to LTL and has an AUC of 0.735 (95% CI = 0.730-0.740). No significant increase in discriminability was observed by the addition of LTL (IDI=0.007; z=0.359, p=0.359).



Supplementary Figure 3.2. Sensitivity analysis of the association between decreased LTL and prospective T2D in DREAM participants versus non-trial (EpiDREAM) participants. All analyses were adjusted for age, sex, hypertension status, waist to hip ratio, physical activity, smoking status, and ethnicity. Het p, χ^2p-value for heterogeneity; Ram, Ramipril; Rosi, Rosiglitazone;

4. Chapter Four: Discussion

4.1. Summary of Findings

The current meta-analysis and systematic review (chapter 2) demonstrates that an association between telomere length and stroke, MI, and T2D is supported by current literature. For each standard deviation decrease in leukocyte telomere length there was a subsequent increase in risk for stroke (OR=1.21, 95% CI =1.06-1.37; I^2=61%), MI (OR=1.24, 95% CI =1.04-1.47; I^2=68%), and T2D (OR=1.37, 95% CI=1.10-1.72; I^2=91%). These findings confirm the hypothesis set out for the first section of the thesis. Substantial heterogeneity was observed for each of the primary outcomes measured. This heterogeneity was not consistently explained (across all outcomes) when stratifying by measurement technique, study design, study size, or ethnicity. Emerging evidence suggests that reasons for heterogeneity may be complex. Variations in DNA extraction methods[1], differences between population-based vs hospital-based controls[2], and the diminishing predictive value of telomere length with age[3] are all factors that likely work in unison to create the heterogeneity present in literature. Due the relatively low number of articles retrieved and only having access to study level data, we were unable to explore complex combinations of factors. Although evidence supporting telomere length as a biomarker for stroke, MI, and T2D is strong, further research is needed to explain heterogeneity.

To further elucidate the relationship between telomere length and cardiometabolic outcomes, we measured telomere length in 19 384 participants

from the INTERHEART and EpiDREAM studies (chapter 3). Age and ethnicity were both factors influencing baseline telomere length across both studies. After adjusting for age, sex, and known modifiable risk factors, a 1 unit decrease in telomere length was associated with an increased risk of MI (OR=2.17, 95% CI=1.74-2.72) in INTERHEART. This relationship was consistent across all ethnic groups (p for heterogeneity = 0.19). Moreover, in EpiDREAM, telomere length was not associated with T2D nor did it appear to predict incident cardiometabolic events such as T2D, CAD, and MACE. Although no association was observed with T2D diabetes at baseline, we cannot exclude the fact that participants in EpiDREAM were selected for being at high risk for diabetes and may have already been experiencing T2D related pathologies. This would undoubtedly attenuate differences between groups and the predictive value of telomeres. Furthermore, due a low number of CVD events (CAD: n=160, MACE: n=167) in EpiDREAM, we likely lacked statistical power to detect associations with incident CVD events.

Together the findings from both studies (chapter 2 and chapter 3) provide insight into the complex relationship between telomere length and cardiometabolic outcomes. Undoubtedly age is the strongest factor influencing telomere length shortening. This is supported by the fact that telomeres naturally shorten with age. Moreover, ethnicity does significantly influence baseline telomere length after accounting for age and sex differences, but the effect of membership in each group on baseline telomere length varies depending on the

study population. MI appears to be the outcome most strongly related to telomere length, even after accounting for known risk factors. This exciting finding indicates that MI may reflect a greater degree of biological aging than other cardiometabolic outcomes such as T2D. Concordant with results from the meta-analysis, the lack of heterogeneity observed amongst effect estimates stratified by ethnicity suggests that results of previously reported studies can be applied to non-Caucasian populations. The lack of significant findings with respect to T2D in EpiDREAM is in contrast to results from the meta-analysis. These discordant findings suggest confounding factors (such as using a population at high risk for diabetes) may attenuate the relationship between telomeres and T2D. Additionally, the lack of significant findings with respect to CVD outcomes in EpiDREAM suggest that much larger sample sizes (with more events) are required to detect associations with telomere length.

4.2. Current Research Implications

The methods and results presented in this thesis have many implications for designing LTL assays, analyzing results, and critically appraising epidemiological literature on telomeres.

Prior to beginning the assay, care must be taken to standardize sample DNA preparation processes as differences in extraction techniques have been shown to result in systematic differences in LTL measurements. We have used an extensively validated method to measure LTL, however a great deal of time is

required to adapt components of the qPCR assay to individual laboratory equipment and reagents. Ideally methods should be optimized so that both telomere and albumin amplification is as close as possible to 100%, the difference between telomere and albumin efficiency is <10%, and the coefficient of variation (CV) between replicates is <10%. Primer selection, qPCR reaction components, and thermocycling conditions can improve amplification efficiency, whereas variation between replicates can be reduced through automating assay setup.

When analyzing results great attention must be paid to ensuring that LTL measurements are reliable. This can be achieved by monitoring the variation in LTL estimates of a control sample placed on each plate run on the real-time PCR. In the analysis stage we used 10% variation as a cut-off point and repeated batches with >10% variation between plates in the control sample. Chance issues with reagents, assay preparation, and running of the real-time PCR instrument are all likely causes of high variation. Moreover, when constructing regression equations during statistical analyses it is important to always adjust for ethnicity and age when identifying associations with telomere length. As we have shown, both factors strongly influence baseline telomere length.

Finally, there are a many aspects of epidemiological studies that can be scrutinized in order to assess quality. As mentioned previously, ensuring that DNA extraction technique is standardized for all samples is crucial as well as

verifying the accuracy and reliability of LTL measurements. For qPCR assays, such as the one used in this thesis, the CV of T/S ratios reported for controls and replicates should be <10%. Studies often misrepresent the accuracy and reliability of their assay by reporting the CV for the telomere and single copy gene amplicon separately. One should also be weary of studies that do not report an association between age and telomere length. Unless the sample size is small (<100) or the range of ages is tight (<20 years), the lack of an age-telomere length association is indicative of poor quality LTL measurements.

4.3. Future Directions

Future studies looking at the association between telomere length and cardiometabolic outcomes are required to confirm results reported in the present thesis. Prospective studies featuring longer follow-up times and a greater number of events would allow for a more accurate assessment of telomere length as a predictor for future cardiometabolic events. This has already been accomplished in a study following approximately 19 838 participants over a 19 year follow-up period for incident MI (n=929)[4]. Studies of this magnitude do not currently exist for other cardiometabolic outcomes such as stroke or T2D. We present the largest prospective investigation of T2D with respect to telomere length in EpiDREAM (n=11 367).

An area of increasing interest is telomere attrition rate[5]. As opposed to assessing an individual's risk for a cardiometabolic event based on a telomere

length measurement taken at one point in time, telomere attrition rate requires two measurements taken over a span of time. This is of considerable interest because even though an individual may have short telomeres, their telomere length may not substantially decrease over a period of time due to healthy lifestyle changes. Therefore, they may be at a lower risk of disease over time compared to another individual who has longer telomeres at baseline, but a greater attrition rate due to an unhealthy lifestyle. Research in to the association between telomere attrition rate and cardiometabolic outcomes is rare most likely because it is difficult to obtain multiple blood samples from participants over a long timeframe.

Innovative genetic study designs may also be used in the future to infer a causal relationship between telomere length and cardiometabolic outcomes. Since genes are not confounded by environmental factors they may be used to study genetic predispositions to short telomere length and their role in increased risk for disease. Recently, a group of 8 single nucleotide polymorphisms associated with short telomere were found to be associated with increased risk of CAD in approximately 37 684 participants from the CARDIoGRAMplusC4D consortium[6]. This finding provides support for a causal relationship between telomere length and CAD. Analyses of similar size (and design) investigating stroke and T2D would be quite informative, but have yet to be conducted.

4.4. Concluding Remarks

Telomeres protect the most precious information living organisms' possess– their DNA. Erosion of telomeres occurs naturally, but can be accelerated due to numerous factors such as oxidative stress and chronic inflammation. As such, telomeres provide information above and beyond chronological age. In relation to many age-related diseases telomere length serves as an exciting new tool to not only better predict harmful events, but also identify situations in which conventional risk factors do not fully explain biological age. With respect to the association between telomere length and cardiometabolic outcomes, great heterogeneity still exists in reported effect estimates. We demonstrate that telomere length may serve as a universal biomarker for MI, however further research is required to understand this relationship biologically and investigate if (and how) it can be utilized in current health care settings to better assess disease risk.

4.5. References

1. Hofmann, J. N. *et al.* Telomere length varies by DNA extraction method: implications for epidemiologic research-letter. *Cancer Epidemiol. Biomarkers Prev.* **23,** 1129–30 (2014).

2. Sanders, J. L. & Newman, A. B. Telomere Length in Epidemiology: A Biomarker of Aging, Age-Related Disease, Both, or Neither? *Epidemiol. Rev.* **35,** 112–131 (2013).

3. Cawthon, R. M., Smith, K. R., O'Brien, E., Sivatchenko, A. & Kerber, R. a. Association between telomere length in blood and mortality in people aged 60 years or older. *Lancet* **361,** 393–5 (2003).

4. Weischer, M. *et al.* Short telomere length, myocardial infarction, ischemic heart disease, and early death. *Arterioscler. Thromb. Vasc. Biol.* **32,** 822–9 (2012).

5. Nakashima, H. *et al.* Telomere attrition in white blood cell correlating with cardiovascular damage. *Hypertens. Res.* **27,** 319–25 (2004).

6. Codd, V. *et al.* Identification of seven loci affecting mean telomere length and their association with disease. *Nat. Genet.* **45,** 422–7, 427e1–2 (2013).

7. Vasa-Nicotera, M. *et al.* Mapping of a major locus that determines telomere length in humans. *Am. J. Hum. Genet.* **76,** 147–51 (2005).