LEUKOCYTE TELOMERE LENGTH, INFLAMMATION AND AGE-RELATED DISEASES

Thesis presented for the degree of Doctor of Philosophy in the

Faculty of Medicine, University of London

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DECLARATION

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

Signed: **Date:** 18/03/2014

ABSTRACT

Background: Aging is a physiological process characterised by a progressive dysfunction in metabolism and impaired tissue repair capacity, eventually leading to organs' and tissue degeneration. Low-grade inflammation is currently considered the main driver of aging. The measure of telomeres length in peripheral leukocytes (LTL) has been suggested as novel and reliable marker of aging. The aim of this PhD was to investigate the association between (LTL), chronic inflammation and common cardio-metabolic risk factors.

Methods: Four studies were conducted: 1) a case-control analysis of 356 cases with periodontitis (PD) and 206 controls to assess the differences in LTL between cases and controls, 2) a cross sectional analysis of 630 individuals with diabetes mellitus investigating the association between PD, LTL and gluco-metabolic factors, 3) a cross-sectional analysis of 1080 adolescents (13–16 years old) to investigate the association between LTL, inflammation and cardiovascular (CV) disease risk factors and 4) a 10 years longitudinal analysis in 2547 women and 2815 men to assess whether LTL predicted cardiac and vascular phenotypes. LTL were measured using a Real Time Polymerase Chain Reaction method in all studies.

Results: Study I demonstrated that increased systemic inflammation and oxidative stress were associated with shorter LTL in cases with PD versus controls ($P<0.05$). Results from Study II confirmed that shorter LTL was associated with severe periodontal inflammation ($p=0.04$), increased endotoxemia and insulin resistance. In Study III LTL was inversely associated with C-reactive protein ($P<0.001$) and fibrinogen ($P=0.001$) in adolescents. Lastly in Study IV a faster rate of telomere shortening between 53 to 60-64 years was associated with subclinical atherosclerosis at 60-64 years ($p=0.006$). All results were independent of traditional CV risk factors.

Conclusions: This PhD programme provides evidence in support of the use of LTL as novel marker of aging and predictor of age-related diseases.

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A special thanks goes to Dr Klelia Salpea who set up the polymerase chain reaction assay for leukocyte telomere length (LTL) measurement used in this thesis, as well as to Professor Steve Humphries, head of the Centre of Cardiovascular Genetics, who allowed me using his laboratory for the LTL assay and provided part of the consumable costs involved in study 1 and 2. Thanks to Mohamed Parkar, Vanita Shah and KaWah Li for their excellent technical support and expertise which allowed me to conduct my experimental analysis with confidence and proficiency.

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CONTRIBUTION TO THIS WORK

The experimental and vascular data described in this thesis are the result of 3 years of collaborative work between the Vascular Physiology Unit, the Centre of Cardiovascular Genetics of the Institute of Cardiovascular Science as well as the Periodontology Unit of the UCL Eastman Dental Institute. All studies included in this thesis are original in concept and design. I performed most of the work outlined in this thesis but I would like to acknowledge the specific contribution of all staff and collaborators.

I have performed all DNA standardizations and leukocyte telomere length (LTL) assays described in studies 1, 2 and 3. In study 2 I have performed part of the DNA extraction (jointly with Dr Nikolaos Gkranias), and performed the lipopolysaccharide and insulin assays (supervised by the senior lab technician, Mr Mohamed Parkar). I performed all reproducibility studies reported in study 1, 2 and 3 for the LTL assay and contributed to improve reproducibility of the LTL assay previously set up in the Centre of Cardiovascular Genetic by Dr Klelia Salpea. I helped with the analysis of the carotid intima media thickness scans reported in study 4. I contributed to all steps of design and conduct, statistical analysis of the fourth study, establishing a fruitful collaboration with the MRC NHSD and Newcastle University. In addition I have analysed and interpreted the results of the all the studies described in this thesis. Statistical support was received by Dr Francesco D'Aiuto (my secondary supervisor), Prof Peter Whincup (St George's University) and Dr Rebecca Hardy (MRC NHSD Unit). Finally, my supervisors Professor John Deanfield, Dr Francesco D'Aiuto and Professor Nigel Klein as well as the external collaborators Professor Steve Humphries and Professor Thomas vonZglinicki had a significant

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PUBLICATIONS RELATED TO THIS WORK

1. **Masi S**, D'Aiuto F, Kahn T, Wong A, Ghosh A, Whincup P, Kuh D, Hughes A, von Zglinicki T, Hardy R, Deanfield JE on behalf of the NSHD scientific and data collection teams. Rate of telomere shortening and cardiovascular damage: a longitudinal study in the 1946 british birth cohort. ***Submitted to Circulation.***
2. **Masi S**, Gkranias N, Li K, Salpea KD, Parkar M, Orlandi M, Taddei S, Patel K, Darbar U, Donos N, Deanfield JE, Hurel S, Humphries SE, D'Aiuto F. Association between short leukocyte telomere length, endotoxemia and severe periodontitis in people with diabetes mellitus: A cross sectional survey. ***Under revision to Diabetes Care (major revision submitted).***
3. Steffens JP, **Masi S**, D'Aiuto F, Spolidorio LC. Telomere length and its relationship with chronic diseases - New perspectives for periodontal research. ***Arch Oral Biol. 2012 Nov 29.***
4. **Masi S**, Nightingale CM, Day IN, Guthrie P, Rumley A, Lowe GD, von Zglinicki T, D'Aiuto F, Taddei S, Klein N, Salpea K, Cook DG, Humphries SE, Whincup PH, Deanfield JE. Inflammation and not cardiovascular risk factors is associated with short leukocyte telomere length in 13- to 16-year-old adolescents. ***Arterioscler Thromb Vasc Biol. 2012 Aug;32(8):2029-34.***
5. **Masi S**, Salpea KD, Li K, Parkar M, Nibali L, Donos N, Patel K, Taddei S, Deanfield JE, D'Aiuto F, Humphries SE. Oxidative stress, chronic inflammation, and telomere length in patients with periodontitis. ***Free Radic Biol Med. 2011 Mar 15;50(6):730-5.***

ABSTRACTS

1. **Masi S**, D'Aiuto F, Kahn T, Wong A, Ghosh A, Whincup P, Kuh D, Hughes A, Hardy R, von Zglinicki T, Deanfield JE. Rate o telomere shortening and cardiovascular phenotypes. ***Poster and oral presentation British Cardiovascular Society 2013.***
2. **Masi S**, Gkranias N, Parkar M, Suvan J, Hurel S, Donos N, Humphries S, D'Aiuto F. Telomeres Length in individuals with Diabetes Mellitus and Periodontitis. ***Oral presentation IADR 2012.***
3. **Masi S**, Salpea KD, Li K, Parkar M, Nibali L, Donos N, Deanfield JE, Humphries S, D'Aiuto F. Systemic inflammation, oxidative stress and shorter telomeres length in periodontitis. ***Oral presentation IADR 2010.***

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ABBREVIATIONS

8-oxodG	8-Oxo-7,8-dihydro-2'-deoxyguanosine
AAP	American Academy of Periodontology
AGE	Advanced Glycation End-product
AHA	American Heart Association
ANOVA	Analysis Of Variance
APC	Antigen Presenting Cell
ASE	American Society of Echocardiography
BAP	Biological Antioxidant Potential test
BPE	Basic Periodontal Examination
CAL	Clinical Attachment Levels
CCD	Charge Coupled Device detector
CDC	Centres for Disease Control and Prevention
CEJ	Cemento-Enamel Junction
CI	Confidence Interval
CMV	CytoMegaloVirus
COX-2	Cyclo-OXygenase-2
CRP	C Reactive Protein
Ct	Cycle threshold
CV	CardioVascular
CVD	CardioVascular Disease
Δ	Delta
ΔCt	Delta Cycle threshold method
ΔΔCt	Delta Delta Cycle threshold method
DDR	DNA Damage Response

dNTPs	deossi-Nucleotide-Tri-Phosphate
d-ROM	Reactive Oxygen Metabolites test
E	Efficiency of PCR amplification
EAE	European Association of Echocardiography
E/A	Ratio between early and late mitral inflow velocity
E/E′	Ratio between trans-mitral Doppler early filling velocity and tissue Doppler early diastolic mitral annular velocity
eGFR	estimated Glomerular Filtration Rate
Eject. Fr.	Left Ventricular Ejection Fraction
ESR	Electron Spin Resonance
F2-IsoP	F2-isoprostanes
Flow-FISH	Flow cytometry - Fluorescence *In Situ* Hybridization
FoxO1	Forkhead-Box O1
GSH	Glutathione
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HKG	HouseKeeping Gene
HMGB	High Mobility Group Box 1 protein
HOMA	Homeostasis Model Assessment
HOMA-IR	Homeostasis Model Assessment index of Insulin Resistance
HPLC	High Performance Liquid Chromatography
HSC	Haematopoietic Stem Cell
IIP	Insulin/Insulin like growth factor-1 Pathway
IGF-1	Insulin-like Growth Factor-1
iNOS	inducible Nitric Oxide Synthase

IFN-α	Interferon-α
IFN-β	Interferon-β
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-13	Interleukin-13
LA Dia	Left Atrial Diameter
LAL	Limulus Amebocyte Lysate
LDL	Low Density Lipoproteins
LOX	Lipoxygenase
LOOH	Lipid hydroperoxides
LTL	Leukocyte Telomere Length
LPS	Lipopolysaccharides
LVM	Left Ventricular Mass
LVMI	Left Ventricular Mass Indexed to body surface area
MDA	Malondialdehyde
MDRD	Modification of Diet in Renal Disease study
MnSOD	Manganese Superoxide Dismutase
mtRS	mitochondrial Radical Species
NCDs	Non Communicable Diseases
NF-κB	Nuclear Factor-κB

NO	Nitric Oxide
NSHD	National Survey of Health and Development
NTC	Null Template Control
O_2^-	Superoxide anion
$^{\bullet}OH$	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
oxLDL	Oxidized LDL
PAMP	Pathogen-Associated Molecular Pattern
PD	Periodontitis
PI3K	PhosphatidylInositide 3-Kinase
PPD	Probing Pocket Depth
Q-FISH	Quantitative Fluorescence *In Situ* Hybridization
qPCR	quantitative-Polymerase Chain Reaction
RAGE	Receptor of Advanced Glycation End-product
REC	Gingival Recession
Ref	Reference gene
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RQ	Relative Quantity
RS	Reactive Species
RT-PCR	Real-time PCR
S	Single copy gene copies
SBP	Systolic Blood Pressure
SD	Standard Deviation
SOD	SuperOxide Dismutase

STELA	Single Telomere Length Analysis
T	Telomere repeats
TAOS	Total AntiOxidant Status
TE	Tris-EDTA buffer
TERC	Telomerase Reverse Transcriptase
TERT	Telomerase RNA Component
TG	Target Gene
Th1	T helper 1 cell
Th2	T helper 2 cell
TLR	Toll-Like Receptor
Tm	melting Temperature
TMB	TetraMethylBenzidine base
TNF-α	Tumor Necrosis Factor-α
TOP	Take-Off Point
tPA	tissue Plasminogen Activator
TRF	Terminal Restriction Fragment
Trx	Thioredoxin
Tsa-1	Thiol Specific Antioxidant 1
UCP2	human UnCoupling Protein 2
UV	UltraViolet light
vWF	von Willebrand Factor
WC	Waist Circumference
WHO	World Health Organization

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1. INTRODUCTION

The medical advances of the 20th century have resulted in dramatic increases in life expectancy worldwide, with developing countries showing the most rapid gains (1;2). The predicted global population of people aged over 60 years is forecast to reach 2 billion by 2050 (3) and this raises concerns about the ability of public healthcare systems to cope (1).

Increased life expectancy does, however, present new challenges to the human body, as it has to compensate for longer environmental exposures than our ancestors used to have. A lifetime of environmental burden may complicate a variety of clinical conditions (4), hence the incidence of age associated illnesses is expected to rise exponentially (5). As a result, global investment in aging research, over the past 25 years, has increased focusing on population studies of healthy aging or frailty and their determinants.

1.1.1 Chronic diseases and their relation with aging

In 2010, the World Health Organization (WHO) reported that noncommunicable diseases (NCDs) including heart disease, stroke, cancer, chronic respiratory diseases and diabetes are the biggest causes of death worldwide, accounting for 63% of all deaths of the worldwide population (6). As mortality for NCDs is particularly high in the elderly, NCDs have been also defined as “age-related” disease. Indeed, it has been estimated that, from the 36 million people who die every year from chronic NCDs, only 9 million are under 60 years of age and 90% of these occurred in low- and middle-income countries (7). As such, predisposition to NCDs of the oldest populations presents an increasing challenge in the most

developed societies, with a growing research interest and financial support provided in the attempt to identify their underlying pathophysiology.

It is widely agreed that increasing chronological age is characterised by an increased vulnerability to stress, due to the decline in homeostatic reserve and secondary to dysregulation in multiple inter-related systems (8-11). The increased vulnerability to stress explains the increased risk of age-related diseases observed with increasing chronological age. However, a large inter- and intra-individual variability within sensory, motor, cognitive and health domains (12;13) are found among individuals of similar age, suggesting that chronological age is a relatively imprecise measure of an individual's functional or health status. The concept of biomarkers of aging was developed to provide more information about an individual's biological health or functional status than chronological age. Biomarkers of aging are quantifiable parameters that reflect biological aging, which potentially can identify those at risk of aging-related conditions, disease, and mortality. Biomarkers could also be used to monitor and evaluate interventions designed to delay the onset or delay the progression of aging-related disorders. For these reasons, much of the biological research into aging over the last few years has focused on the identification of reliable markers which could inform on the risk of developing age-related diseases (14-16).

According to the American Federation of Aging Research, a reliable biomarker of aging should (17;18):

1. Predict the rate of aging. In other words, it would tell exactly where a person is in their total life span. It must be a better predictor of lifespan than chronological age.
2. Monitor a basic process that underlies the aging process, not the effects of disease.

3. Be able to be tested repeatedly without harming the person. For example, a blood or an imaging tests.
4. Be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans.

In a 2005 review of the in vitro and in vivo evidence, von Zglinicki and Martin-Ruiz (19) found that small segments of DNA which cup the end of chromosomes, known as telomere length, satisfied several criteria for a biomarker of aging, as it changes with age, has high inter-individual variability, is linked to basic biology, and is associated with the risk of aging-related disease and mortality. The authors acknowledged that the majority of the evidence was cross-sectional and that many studies were underpowered. For a proper validation of telomere length as marker of aging and aging-related disease, however, it remained to be proved that telomere length biology is influenced by the same factors known or suggested to be in the causal pathway for aging and aging-related disease.

Several epidemiological studies have shown that chronic inflammation and oxidative stress exposure are the main drivers of aging and aging-related diseases. Chronic inflammation is currently considered the major risk factor for cardiovascular disease (CVD), cancer, type 2 Diabetes, Alzheimer's and Parkinson's diseases. Biologically, it has been proved that chronic inflammation and oxidative stress exposure increase the risk of aging and aging-related diseases, by causing multiple cumulative cellular injuries and reducing cellular ability to repair and regenerate. The mechanisms by which inflammation and oxidative stress promote cellular aging include: (i) cumulative oxidative damage due to increased generation of free radicals (20;21); (ii) cumulative DNA damage coupled with declines in the ability to repair (22); and (iii) cellular senescence, a state in which cells no longer divide (23). The

ideal bio-marker capable of predicting predisposition to age-related diseases should be able to inform on all these biological processes.

The aim of this thesis was to further validate the measure of telomere length as possible marker of aging and aging-related diseases, by exploring its cross-sectional relationship with chronic oxidative stress and inflammatory burden and aging phenotypes.

1.2 TELOMERES

1.2.1 Telomere biology and cellular aging

Research into cellular senescence has always attracted interest in medicine. The dominant view of the early 20th century, highlighted in Alexis Carrel's publication 'on the permanent life of tissues outside the organism' (24), was that cells could grow indefinitely. It was not until 1962, that Leonard Hayflick challenged this dogmatic view by demonstrating how somatic cells had a limited capacity for replication (25). This phenomenon has since been termed 'the Hayflick limit', nowadays referred to as replicative senescence. A few years later, Elizabeth Blackbourne discovered an intracellular sensing mechanism that limits cell lifespan in culture: telomere length (26). After this discovery, several experimental and human clinical studies demonstrated clearly that the measure of telomere length stands at a junction of critical processes underlying human chromosome integrity and vulnerability to chronic illnesses including cancer, CVD and, more generally, age-related diseases. The importance of Dr Blackbourne's discovery was recently recognized worldwide as she has been awarded of the Nobel Prize in Physiology and Medicine in 2009 (27).

1.2.1.1 Telomere structure

Telomeres are ribonucleoprotein complexes capping the ends of chromosomes and are essential for chromosome protection and genomic stability. Without telomere capping, chromosomal ends could be recognized as sites of DNA double-strand breaks, leading to chromosome degradation by DNA repair mechanisms. Furthermore, telomere capping prevents end to end fusion of chromosomes during

cell replication (27-29). The telomere sequence consists of stretches of repetitive DNA (hexamers) with high guanine content (30;31).

Figure 1.1 Simplified schematic diagram of telomere structure.
Telomeres are located at the ends of linear chromosomes. In humans, they are composed of hundreds to thousands of tandem DNA repeat sequences: hexameric TTAGGG in the leading strand and CCCTAA in the lagging strand. Additional protective proteins are also associated with telomeric DNA and are collectively called shelterin (TRF1, TRF2, TIN2, POT1, TPP1). The 3′ end of the telomeric leading strand terminates as a single-stranded overhang, which folds back and invades the double-stranded telomeric helix. Adapted from Kovacic JC et al. Circulation 2011;123:1650-1660.

In humans, the telomere terminus consists of 4 to 15 kbp of the hexanucleotides 5′-TTAGGG-3′ and ends in a G-rich 3' overhang (G-strand overhang) (32;33). The G-strand overhang can fold back and invade the double-stranded region of the telomere, thereby generating a looped structure, known as the telomere loop or T-loop (34). The structure hides the 3' end from telomerase as well as from DNA repair and degradation activities, representing, therefore, a primitive mechanism for telomere protection (35).

Telomeres are bound by major protein complexes, the most important of which is known as shelterin (or telosome). Shelterin encompasses the Pot1-TPP1 heterodimer, the telomere-binding proteins TRF1 and TRF2, and the interacting factors Rap1 and Tin2 (36). Shelterin has a key role in regulating telomere length as it stimulates telomerase (the enzyme responsible for telomere elongation) and confers to telomeres the ability to acquire a T-loop conformation (37;38).

1.2.1.2 Telomere length regulation

During each cell division, in most proliferating cells, there is a gradual decrease (~20- 200bp) of telomere length, as a consequence of the end replication problem (Figure 1.2) (39).

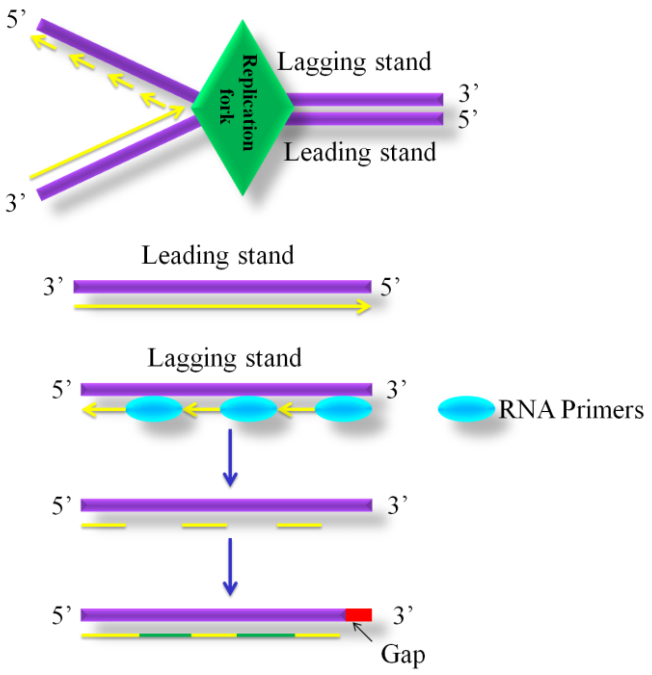


Figure 1.2 End-Replication Problem.
DNA polymerase requires an RNA primer to initiate synthesis in the 5'-3' direction. At the end of a linear chromosome, DNA polymerase can synthesize the leading strand until the end of the chromosome. In the lagging strand, however, DNA polymerase's synthesis is based on a series of fragments, called Okazaki, each requiring an RNA primer. Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primer event. The result is an end-replication problem in which sequence is lost at each round of DNA replication.

DNA polymerase can synthetize new DNA only in 5'-3' direction. Therefore, while the leading strand can be fully replicated until the end of the chromosome, as the synthesis proceeds in 5'-3' direction, in the lagging strand, the synthesis of new DNA depends on the addition of a series of fragments, called Okazaki, each requiring a RNA primer. When these RNA primers are removed, it is impossible for the DNA polymerase to synthesize the lagging-strand sequence that is complementary to the small region at the end of the chromosome (which is at least as large as an RNA primer). The net result of this process, known to as the end-replication problem, is the elimination of some telomeric repetitions with each round of cell division, resulting in a progressive chromosome shortening. Cell proliferation is therefore considered the most important cause of telomere shortening in all human tissues, and the presence of short telomere length in the elderly can be interpreted as the accumulation of cell divisions associated with life time tissue renewal (40).

Aside from the end-replication problem, however telomere shortening can be further accelerated by a number of DNA damaging agents. Among these, exposure to high levels of reactive radical species (RS) has been the most widely studied (19;33;41-45). Due to their high guanine content, telomeres are highly sensitive to hydroxyl radical damage, which results in single-strand breaks of the telomere sequence. Damage can occur either as a direct effect or as an intermediate step in the repair of oxidative base modifications (8-Oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxodG) (46). While similar mechanisms of oxidative DNA damage have been described in other “guanine-rich” genomic regions, telomeres have been reported to be deficient in the repair of single-strand breaks (47). Subsequently, they appear to be especially vulnerable to the accumulation of RS-induced 8-oxodG DNA-strand breaks (48).

The main compensatory mechanism of telomere shortening is the action of a specific enzyme called telomerase, which promotes telomere elongation (49). This enzyme contains two molecules, the telomerase reverse transcriptase subunit (TERT) and the telomerase-associated RNA component (TERC), as well as one molecule of dyskerin (50), acting as stabiliser of the complex (Figure 1.3).

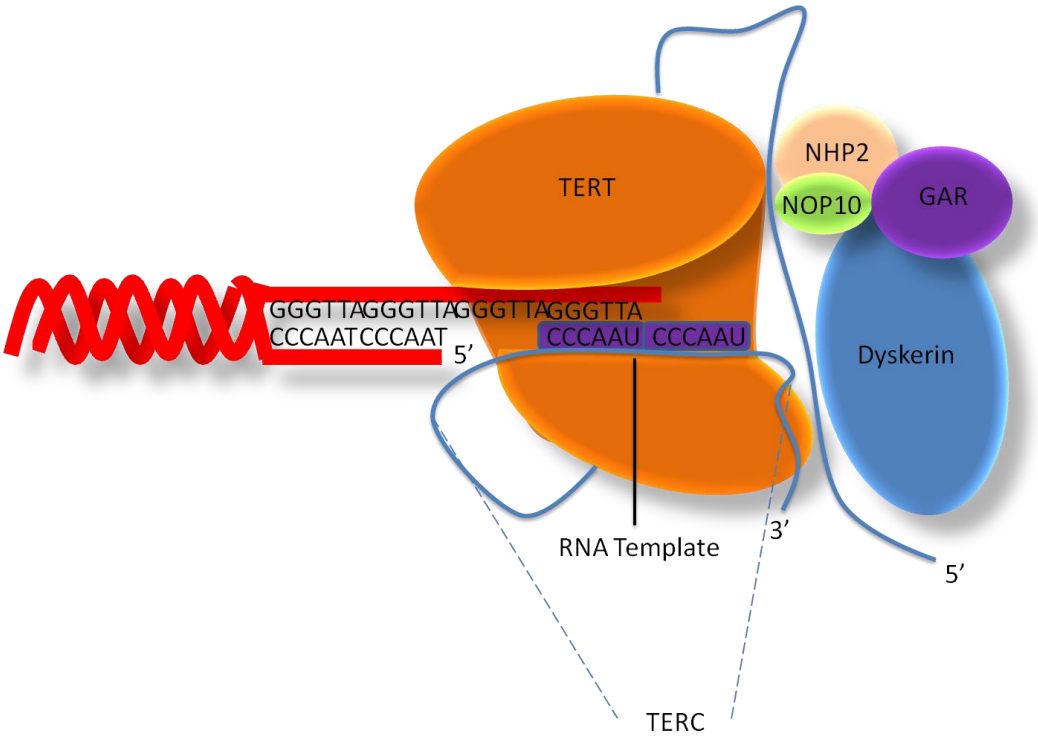


Figure 1.3 Structure of telomerase.
Telomerase is the main mechanism for telomere length elongation. The telomerase enzyme consists of two molecules of the TERT subunit and two molecules of the associated TERC, which contains the template for the addition of new telomeric repeats. In addition, telomerase contains one molecule of dyskerin, a protein that stabilizes the telomerase complex. Telomerase recognizes the 3' end of the G-rich telomere strand and adds telomeric repeats de novo.

In the majority of human tissues, telomerase is highly expressed during embryonic development, with a rapid down-regulation after birth. While certain cells including those in the hematopoietic lineage maintain the ability to activate telomerase in post-natal life, this is insufficient to prevent telomeres from shortening with aging (51;52).

Importantly, when telomeres become critically short, they lose the ability to form the T-loop and are recognised as sites of DNA damage. As this phenomenon cannot be repaired by endonuclease and DNA polymerase, it triggers a persistent activation of the DNA damage response (DDR), which leads to cellular senescence and/or apoptosis (53;54). These latter conditions are characterized by a permanent arrest of the cellular cycle through activation of the p53 and p21 checkpoints (55) (Figure 1.4).

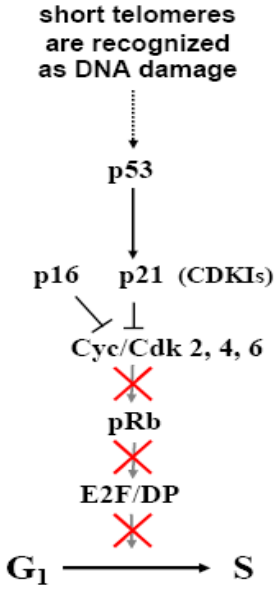


Figure 1.4 Critically short telomeres stop cell replication by activation of DDR.

Furthermore, senescent cells lose most of their metabolic activity whilst apoptotic cells are rapidly removed from tissue by tissue resident macrophages. In both cases, these cells can no longer contribute to the metabolic activity of tissues and their accumulation is considered a cause of age-related deterioration of tissue function and reduced ability to respond to acute stress (56). As such, shortened telomeres might be indicators of tissue aging, low stress resistance and increased risk of disease.

1.2.2 Telomere length in humans: from biology to epidemiology

1.2.2.1 Epidemiological studies

The hypothesis that shortened telomeres length predicts age-related disease was initially proposed in humans in 2000, when short telomeres in peripheral circulating cells were associated with increased risk of cerebrovascular disease (57). A few years later, Cawthon et al. published a study reporting an association between short leukocyte telomere length (LTL) and increased mortality rate (58). In a cohort of

143 normal unrelated individuals over the age of 60 years, Cawthon documented that individuals with shorter LTL exhibited a threefold and eightfold increase in mortality by CVD and infectious disease, respectively. Subsequently, several large epidemiological studies confirmed the association between short LTL and increased risk of CVD morbidity and mortality (58-64). Moreover, new strong associations were described between LTL and indices of obesity/insulin resistance (62;63;65-67), cancer (68;69), dementia (57;70), and a host of other age-related diseases.

However, the evidence produced in these reports was inconclusive. Indeed, several studies including in elderly populations could not find associations between short LTL and cardiovascular (CV) mortality (table 1.1)

Table 1.1 Studies which did not find association between telomere length and mortality

Studies	Population	Age	Method	Results
Martin-Ruiz et al. (71)	N= 598 (Leiden Study population)	>85 yrs old	qPCR	Telomere length did not predict mortality or incidence of dementia
Bischoff et al. (72)	N=812 (Longitudinal Study of Aging Danish Twins + Danish 1905 Cohort Study + Longitudinal Danish Centenarian Study)	>70 yrs old	TRF	LTL did not predict mortality following adjustment for age
Houben et al. (73)	N=203 (Zutphen Elderly Study)	>70 yrs old	qPCR	LTL did not predict all cause, cancer or CVD mortalities
Strandberg et al. (74)	N=622 (Helsinki Businessmen Study)	>70 yrs old	qPCR	LTL did not predict mortality

qPCR = Quantitative Polymerase Chain Reaction; TRF: Terminal Restriction Fragment

Furthermore, several reports could not replicate the associations between LTL and common CV risk factors, including dyslipidaemia, hypertension (75) and parameters of insulin resistance (76;77), nor with the incidence of cerebrovascular disease (78) or coronary heart disease (79).

Several reasons could account for these discrepancies.

a) Exploratory epidemiologic surveys often do not correct for multiple variables, making it difficult to understand the relative contribution of different factors on cross-sectional and longitudinal measures of LTL.

b) The majority of published studies are underpowered, due to high inter-individual variation in LTL at birth and thereafter. The high inter-individual variation in LTL at birth and the effects of genetic factors, sex, and environmental pathogens on the rate of telomere attrition give rise to considerable variation in age-adjusted LTL. It follows that large numbers of individuals are required in observational studies (i.e. cross-sectional) to uncover potential links between LTL and age-related disease.

c) The use of samples from the latter stages of the life span may lead to a "survival effect". LTL has been linked with aging and diseases of aging, so that individuals with aging-related disorders or those prone to age faster appear to have relatively short LTL. It follows that (if the observations are correct) these individuals are more likely to die at a younger chronological age than their peers, leaving older survivors with telomeres which are relatively longer than expected in the general population. Thus, cross-sectional observations may over-estimate telomere length in the elderly and there may be less variation within this group. Such a reduced inter-individual LTL variation challenges the accuracy of current LTL assays, increasing the difficulties in detecting mortality differences related to LTL. For this reason,

case–control disease studies of the elderly have to be closely matched for age and results from extreme elderly cohorts interpreted with caution (71;72).

d) Finally, the methods used to measure telomere length may not be sufficiently precise to decipher the links between leukocyte telomere dynamics and aging, diseases of aging, and longevity in humans.

1.2.2.2 LTL assays: Southern blot vs quantitative-polymerase chain reaction

Over the last 20 years, researchers have developed several methods to measure telomere length, including Southern blot, quantitative fluorescence *in situ* hybridization (Q-FISH), flow cytometry (flow-FISH), quantitative-polymerase chain reaction (qPCR) and single telomere length analysis (STELA). Table 1.2 reports pros and cons of the different methods. While Q-FISH, flow-FISH and STELA assays are the most accurate methods and can specifically measure individual telomeres per each chromosomal arm, they require intact cells for analysis, limiting their application to epidemiological and longitudinal investigations (80). Furthermore, most of these assays are laborious, time consuming and operator dependent when compared to other semi-automated techniques. This strongly affects reproducibility of the assay and limits comparison of results between different laboratories. It is not surprising, therefore, that the two major methods currently used to measure telomeres in epidemiological/clinical research are Terminal Restriction Fragments (TRFs) length analysis (31) by Southern blot (81) and qPCR (82;83).

Table 1.2 Advantages and limitations of the most common telomere length assays

Assay	Advantages	Limitations
Southern Blot	• Absolute quantification of telomere length (in base pair, bp) • Reference technique • Good reproducibility in expert laboratories	• The terminal restriction fragment length includes also the sub-telomeric region • High operator dependent • Requires good quality (DNA needs to be tested for integrity before use) and high amount of DNA (1µg) • Expensive, cumbersome, labor intensive • Quantification of smear patterns in autoradiograms is prone to error and data is often reported as a mean of the TRFs, yet the TRF distribution might not always be Gaussian.
qPCR	• High throughput (easily applicable to large epidemiological studies) • Cheap • Less operator dependent (can be completely automated) • Requires low amount of DNA (even 60ng are enough) and maintains its accuracy on long term stored samples (it is not necessary to test DNA integrity before the assay) • Assessment of the telomeric region alone (without sub-telomeric fragment)	• Relative quantification → does not provide absolute telomere length measures in bp) • High variability in the devices and methods used by different labs → makes difficult to compare results of different studies) • High risk of artefacts → every minute change in the efficiency of the amplification might be amplified during each thermal cycle, resulting in substantial variations in accumulating products, with considerable impact on the final result
FISH	• High accuracy • Can provide the measure telomere length in different cell phenotypes • Possible with low amount of cells • Opportunity to co-stain multiple intracellular markers together with telomeres – IMMUNOFISH	• Time consuming • High operator dependency • Expensive • Requires complex post-acquisition analysis • Requires fresh cells • Should be performed only on cells in metaphase (although recently this problem has been partially solved)
Flow-FISH	• High accuracy • Able to target the measure of telomere length in specific cell phenotypes • Does not require cells in metaphase • Opportunity to co-stain multiple intracellular markers together with telomeres	• Requires high amount of cells (at least 3 $x10^6$) • Difficult on stored blood → results can be affected by storing procedure • Expensive • Time consuming • Complex post-acquisition analysis • Does not provide an absolute telomere length quantification (although this information can be recovered using specific fluorescent beads)
STELA	• High accuracy • Allows measuring telomere length on specific chromosomes (i.e. chromosome 17 or chromosome X) • Enable the study of alternative pathways related to telomere erosion/elongation	• Time consuming • High operator dependent • Expensive • Requires good quality and high amount of DNA • Should be performed on fresh blood

Southern blot was the first technique used to measure telomeres length and is performed on the terminal restriction fragments (TRFs), generated by subjecting genomic DNA to restriction enzymes (e.g., Hinf I/Rsa I, Hph I/MnI I). The processed samples are resolved on gels and hybridized to a telomeric probe ([CCCATT]n). Autoradiographs are subsequently scanned and subjected to densitometric analysis. Despite being the most frequently used technology to quantify TRF, this approach has numerous drawbacks (see Table 1.2), the most important of which are the large amounts of DNA required for analysis, the high costs of reagents and the high operator dependency. Furthermore, the method provides only an estimate of the length of telomere repeats, as the restriction enzymes generate TRFs that consist of a mixture of canonical (strictly TTAGGG) repeats of telomeres and some non-canonical (sub-telomeric) sequences proximal to the telomeric region. As sub-telomeric sequences can vary in length depending on the last restriction site at a given chromosome arm (84), this increases the heterogeneity of the TRFs, masking the real length of telomeric repeats (85).

In 2002, Cawthon developed a novel method to measure telomere length using qPCR (82). Previously, the measurement of telomere by PCR amplification (using oligonucleotide primers, TTAGGG and CCCTAA) was considered impossible due to the high risk of hybridization between primers. To overcome this problem, Cawthon modified the oligonucleotide primers. They consist of a repeated pattern of six bases containing four consecutive paired bases followed by two mismatched bases (82). This modification prevents the primers hybridizing with each other, leaving the PCR-amplified telomere products free of primer-derived products. Thus, with the qPCR-based assay, final telomere length can be determined by the ratio between amount of telomere repeats (T) and that of single copy gene copies (S) in each sample (T/S

ratio), compared to a T/S ratio of a reference DNA sample. The introduction of a reference DNA sample has allowed standardization of the results across separate PCR runs (82).

The qPCR technique is, relatively simple, cheap and of high throughput when compared to the TRF analysis. Moreover, it requires smaller amounts of DNA (~60 ng) and, as it measures telomere repeat content rather than absolute telomere length, and can be safely used to analyse degraded DNA samples (80). Finally, the primer design also counteracts the contribution of the sub-telomeric region as each primer is complementary specifically to telomeric repeats. One of the main limitations of this method however is that it only renders relative (T/S ratio) and not absolute (bp) values of telomere length (80). Researchers have tried to overcome this problem by using a conversion factor to transform the T/S ratio into kilobases. This factor is derived from the slope of a regression line describing the relationship between the T/S ratios and the mean TRFs in 'reference' samples (58). As a host of variables are intrinsic to the PCR methods and the DNA samples used in different laboratories, the conversion factor may differ in individual studies and in DNA samples derived from different groups, e.g., old versus young people. However, the successful introduction of an oligomere standard to measure absolute telomere length (71;86) has partially circumvented this problem. Another major limitation of the qPCR based telomere length assay resides in its high risk of generating artefacts. Indeed, every minute change in the efficiency of the T and S reactions may be amplified during each thermal cycle, resulting in substantial variations in accumulating products, with considerable impact on the T/S ratio.

In conclusion, while Southern blots and qPCR have specific limitations, the simplicity, low cost and high throughput ability, make them both reliable in

measuring LTL, with the qPCR technique being the preferable choice for large epidemiological studies due to its lower operator dependency.

1.2.2.3 LTL heritability and dynamics in humans

LTL dynamic is complex in humans. Age-adjusted LTL is highly variable because of inter-individual differences in telomere length at birth and the rate of telomere attrition thereafter (87-90). In principle, a person with faster age-dependent telomeres erosion may not always have shorter telomeres than his age-matched peer (i.e. if the former was born with longer telomeres). Although robust telomerase activity in fetal tissues (91) results in high synchronization of telomere length in all different somatic cells within a specific newborn, LTL variation among new-borns is known to be as wide as that found in adults (~4 kb) (89). Much of this variation is considered to be genetically determined (92-102). Indeed, data from twin studies estimates, the heritability of LTL to be between 0.36 and 0.84 (103).

Currently, two main factors have been identified as accounting for most of the heritability of telomere length: gender and paternal age at conception.

Nawrot et al were the first to describe a possible X-linked inheritance of LTL (97). They reported high synchronization of LTL between fathers and daughters, mothers and sons and daughters and among siblings, but found no association between telomeres of spouses or those of fathers and sons. Additionally, Akkad et al. observed a robust correlation of LTL between mothers and their newborns (104). While this evidence suggests the presence of an X-linked imprinting in LTL, Nordfjäll et al suggested a paternal mode of heritability (99;100). These results were later confirmed by Njajou who described a greater paternal heritable effect of LTL compared to the maternal counterpart (98). Although it remains unknown which maternal or paternal

mechanisms are involved in defining telomeres length heritability, this evidence clearly supports a strong influence of genetic factors on LTL at birth.

Another intriguing observation made with regards to telomere length inheritance concerns the effect of paternal age on offspring's telomere length. While the shortening of telomere length with age is well established in most proliferating tissues, sperm telomere length is an exception, as older men have longer sperm telomeres (105;106). This finding is likely to be determined by the high activity of telomerase in the testes (107;108). As offspring inherit half of their chromosomes from paternal sperm, it is not surprising that offspring of older fathers tend to have longer telomeres (105;106;109). The effect of paternal age at conception on their children was firstly observed by Unryn et al. in 2005 (109), and it has been since confirmed in several large cohort studies (105;106;110). Intriguingly, a recent investigation demonstrated that the age-related telomere length increase in sperm could lead to multigenerational, cumulative, and thus more biologically significant lengthening or shortening of telomeres (111).

Several studies have investigated changes of LTL after birth. Cross-sectional and longitudinal analyses suggest that LTL shortens at a much faster rate (~200 kb/year) during infancy and early childhood compared to adulthood (~30-40 kb/year) (112). This higher rate of LTL shortening has been attributed to the rapid somatic growth and expansion of the hematopoietic stem cell (HSC) pool in the bone marrow. Indeed, at any age, LTL mirrors the length of telomeres in HSCs (113;114), and the higher demand for cell replication in the HSC pool is ultimately reflected in a faster rate of telomere shortening in peripheral leukocytes, their "daughter" cells. During adulthood, there is a progressive reduction in LTL shortening rate.

This is mainly due to:

a. a shift towards a prevalent housekeeping HSC activity, necessary only to sustain its own self-renewal and to replace HSC's lost through repeated replicative cycles;
b. the inverse relationship between baseline LTL and rate of telomere length shortening, which makes shorter telomeres less prone to attrition than their longer counterparts (see the paragraph *Unexpected findings from longitudinal measurements of LTL)*.

1.2.2.4 LTL dynamic challenges LTL assays

The reduced rate of LTL shortening in adults represents a major challenge for current techniques used to measure LTL in humans, especially for longitudinal evaluations of telomere dynamics in large epidemiological studies. The reproducibility of the LTL assay should be as high as possible to capture small changes in the rate of telomere attrition between individuals exposed to different environmental factors. The most reliable way to assess reproducibility of LTL assays in large-scale epidemiological studies is to run a subset of randomly selected samples on two different occasions and calculate the inter-assay coefficient of variation between the two set of measurements (though this could potentially underestimate the true coefficient of variation based on separate DNA extractions).

An example of this problem is elucidated below.

Example

The average of duplicate measure of TRF length in subject A is 7,500bp

LTL assay coefficient of variation is ~2%

Question: What is the potential error in estimating the biological age of subject A using such a LTL assay?

1. Coefficient of Variation = (SD/Mean)
2. SD = (Mean*coefficient of variation) = (7,500*0.02) = 150bp (0.15 kb)
3. Telomere attrition is ~30 bp a year
4. Error in estimating the biological age = 150bp/30bp a year = 5 years

Therefore, using a method with only 2% of coefficient of variation it is possible to make an error in the biological age of the individuals of ~ 5 years.

However, the coefficient of variation is calculated using standard deviation which, in the case of normally distributed data, defines the variability around the mean of only the 68% of the total population. Therefore, only 68% of the measured values of LTL will fall within the estimated coefficient of variation of 150bp.

To further highlight the relevance of the problem, we can calculate the 95% coefficient interval for the measurement of 7,500 bp. This parameter provides a better estimate of the accuracy of a measurement, as it defines the range of values within which, changing the sample of interest, the LTL measurement, will fall with a probability (confidence) of 95%. In other words, repeating the assay on multiple samples, the calculated confidence interval (which would differ for each sample) would encompass the true population parameter 95% of the time.

Therefore, proceeding with the calculation:

1. 95% coefficient interval (CI): Mean±1.96*σ/√n
2. 95% CI: 7,500±1.96*150/√1 = 7.21–7.79 kb

The difference between these two extremes corresponds to a deviation of 20 yrs in putative biological age.

This example contributes to explain the relatively low number of studies in which longitudinal measures of LTL has been reported so far, although longitudinal

evaluations of telomere dynamics are obviously a better choice than cross-sectional analysis to study LTL dynamic and its determinants in humans.

1.2.2.5 Unexpected findings from longitudinal measurements of LTL

A small number of epidemiological cohort studies have reported information on longitudinal variation of LTL dynamics (71;87;114-118). The most consistent finding originating from these reports is a paradoxical elongation of LTL in a subset of individuals despite their advancing age (~20% to 30%, depending on the study) (87;116;117). No clear mechanisms have been identified to explain this evidence, but several hypotheses have been formulated.

i) It has been suggested that the duration of follow-up of these observational studies was not sufficient to counteract the intrinsic variation/error of the technique used to quantify LTL, resulting in an unreliable detection of the LTL attrition rate. As the rate of LTL shortening is extremely low, short follow up will result in small differences in the measure of LTL between the two time points. Such small differences are likely to fall within the intrinsic variability of the technique used to measure LTL. The only study confirming this hypothesis is that of Chen et al., including a subgroup of 271 participants of the Bogalusa Heart Study (119). Using southern blots they measured LTL at three consecutive time points (S1, S2, S3) with variable intervals (gap between S1 and S2 of 5.8 years, between S2 and S3 of 6.6 years and between S1 and S3 of 12 years) and demonstrated that LTL lengthening progressively decreased with longer follow up periods (between S1 and S2: 14.4%, between S2 and S3: 10.7% and between S1 and S3: 1.5%). Furthermore, using residual DNA aliquots of the same individuals who displayed telomere elongation,

they repeated the LTL measurements after 6 months and the paradoxical elongation was only confirmed in 1/3 of the samples. Whilst these data suggest that telomere elongation could be an artifactual finding dependent upon the low reproducibility of the assay used to measure LTL, it should be noted however that telomere elongation has been truly observed in all longitudinal studies of LTL. Additionally, the percentage of individuals with telomere lengthening is similar among all studies, despite the substantial differences in the reported inter-assay coefficient of variation and duration of follow up (87;114-118).

ii) A second hypothesis suggests that the occurrence of acute infections can eventually affect the relationship between LTL and stem cells telomere length between baseline and follow up observations (87). Age-dependent LTL shortening would largely mirror telomere attrition in hematopoietic stem cells and progenitor cells, assuming that telomere shortening downstream of hematopoietic stem cells/progenitor cells is relatively constant. However, this phenomenon might not hold all the time. For instance, acute infection might transiently increase proliferation of peripheral mononuclear cells, resulting in a temporary increase in the difference between LTL and telomere length in hematopoietic stem cells/progenitor cells. This transient effect would not shorten telomere length in hematopoietic stem cells/progenitor cells over the life course of the individual, but it might affect LTL results in a longitudinal study of short duration.

iii) An alternative hypothesis relates to the status of hematopoietic stem cells within bone marrow niches. Indeed HSC residing in the osteoblastic niche are largely quiescent and their mobilization into the vascular niche promotes their

transformation into proliferative HSC's (120). It is possible that, in individuals with telomeres elongation, a group of quiescent HSC are mobilized into the vascular niche between baseline and follow-up examinations, resetting LTL above its length at the baseline examination.

Although all the above-listed hypotheses are biologically plausible, they may be insufficient to account for the relatively high and consistent percentage of individuals presenting with LTL elongation in a number of observational studies. It could well be that LTL elongation is a true biological phenomenon, with a potentially important but still obscure clinical meaning. Indeed, in a sample of 236 randomly selected Caucasian participants from the MacArthur Health Aging Study (aged 70 to 79 years), Epel et al. showed different trajectories of LTL (i.e. telomere shortening, maintenance and elongation) were associated with differences in CV mortality rate (121). Individuals with telomere elongation or stability had better CV outcome compared to those with telomere shortening. However, the relatively short follow-up of the study (only 2.5 yrs) raises several doubts on the ability of the qPCR based telomere length assay used in this study to capture the expected small difference in trajectories of telomere attrition.

It has been repeatedly reported that the rate of LTL shortening strongly depends upon the baseline measure of telomere length, with shorter LTL at baseline associated with lower rates of LTL attrition at follow up (87;116-118). This finding is unlikely to be dependent upon the reduced sensitivity of the LTL assay, as it has been documented in all studies in which LTL was measured in a longitudinal fashion. Remarkably, several studies confirm a possible feedback regulation of the rate of LTL

shortening. Indeed, whilst telomere length is variable among different chromosomes (122) as well as between homologous chromosomes (123), it has been shown that it shortens faster in the homologous chromosome with the longer telomeres. The most likely mechanisms of this phenomenon include:

a) oxidative stress exposure (124;125), because longer telomeres are bigger targets for free radicals, which attack the G triplets on the sequences.
b) residual telomerase activity, which would preferentially add telomeric repeats onto the shortest telomeres as they are unable to adopt the T-loop conformation (123).

An easier alternative explanation of this finding could be far from a biological phenomenon but represent a mere consequence of the mathematical coupling of the baseline and follow-up measure of LTL. Mathematical coupling is the effect that occurs when one variable directly or indirectly contains the whole or part of another, and the two variables are analysed using standard correlation or regression techniques (126-130). The most commonly observed form of mathematical coupling in medical research occurs by addition, subtraction, multiplication, or division of one variable by another (126). This is frequently encountered when one is investigating the change in outcome during follow up (i.e. following a specific intervention to test the effectiveness of a particular treatment) in relation to initial or baseline value (i.e. the outcome prior to the intervention). This is because the baseline is part of the change, and thus change and baseline are *coupled* algebraically (hence the term mathematical coupling). This then affects the statistical procedure of testing the null hypothesis, which is central to the interpretation of the correlation or regression analysis. Where a statistical analysis suffers from mathematical coupling, the null hypothesis being tested—that the coefficient of correlation or the slope of regression is zero—becomes

erroneous (131). Interpretation of results, and thus any purported evidence, from studies that analyse change with respect to baseline in this way are therefore erroneous and the conclusion(s) reached become questionable.

1.3 INFLAMMATION

1.3.1 History

The recognition of inflammation dates back to antiquity. The first to define clinical symptoms related to the inflammatory response was the Roman doctor Cornelius Celsus in the 1st century AD. As documented by Celsus in his treatise *De Medicina*, the ancient Romans understood that tissue response to injury could be summarized by four principal signs: *rubor* (redness due to hyperaemia), *tumor* (swelling, caused by increased permeability of the microvasculature and leakage of proteins into the interstitial space), *calor* (heat associated with the increased blood flow and metabolic activity of the cellular mediators of inflammation), and *dolor* (pain, in part due to changes in the peri-vasculature and associated nerve endings) (132). It was only in the 19th century, however, that Augustus Waller and Julius Cohnheim discovered the physiological basis of these four characteristic signs (133). They proposed that acute inflammatory responses were characterized by emigration of leukocytes from the blood vessels, accompanied by other changes in the local vasculature such as vasodilation and leakage of plasma into the interstitial space. In 1858, Rudolph Virchow added a fifth postulate of inflammation termed *functio laesa*, to identify organ dysfunction which develops during the inflammatory process (132). While the initial signs only apply to acute inflammation accompanying wounds and infections, *functio lasesa* universally accompanied all inflammatory processes. The introduction of the concept of cellular phagocytosis and of the theory of cellular immunity by Elie Metchnikoff in the late 19th represented the next milestone in our understanding of the inflammatory process. Through watching protozoa engulf particulate matter and examining blood leukocytes ingest foreign bodies she

discovered the phagocytosis process. Based on these findings, she emphasised the beneficial aspects of inflammation, pointing out the key role of macrophages and neutrophils in host defence and maintenance of tissue homeostasis (134). Subsequent milestones included the identification of different classes of serum components as crucial regulators of the inflammatory response.

Over the recent decades, the introduction of high sensitivity assays for inflammatory markers has greatly increased the understanding of the pathophysiological mechanisms involved in inflammatory responses. Inflammation not only acts as first line of defence of the human body against external aggressions but, more generally, represents an adaptive response of the human body to transient or persistent perturbations in its internal homeostasis.

1.3.2 Acute and chronic inflammatory responses safeguard the body's internal homeostasis

Homeostatic control mechanisms ensure that the internal environmental and vital parameters (such as glucose and oxygen concentrations) are maintained within physiological ranges defined at specific set points (135). External aggressions or internal perturbation of tissue homeostasis can cause deviation in some parameters beyond the physiological range, resulting in acute cellular or tissue stress that elicit in a transient adaptation to the new environment (acute inflammation). As a result, acute inflammation represents a short-term adaptive response to transient abnormalities. Its 'purpose' is to remove the source of the disturbance, ultimately restoring functionality and homeostasis to the tissue. The ability to detect clinically the ongoing inflammatory response (i.e. using traditional inflammatory markers) is dependent on the nature and the degree of the underlying tissue malfunction. For example, very

mild stress might only cause local alterations that can be easily handled by tissue-resident inflammatory cells (mainly macrophages and mast cells). More extensive dysfunction or damage, by contrast, may require recruitment of additional leukocytes and plasma proteins, causing a transient increase in the systemic levels of inflammatory cells and/or markers.

If the abnormal conditions are sustained, more prolonged adaptive changes are required (chronic inflammation). The ongoing inflammatory state shifts the system to different and pathological set points that are better suited to deal with the extreme or persistent abnormal conditions. These adaptive changes generally occur at the expense of many other physiological processes and cannot be sustained without adverse side effects. Therefore, while an adaptive shift of endogenous set points can provide short-term benefits, in a chronic phase it can become maladaptive, causing further alterations to the body's homeostasis. This explains why, although chronic inflammatory responses are normally less aggressive than acute inflammatory reactions, they are currently considered the major contributors to the medical burden of industrialized societies (136). The decline in insulin sensitivity of skeletal muscle as a result of systemic inflammation provides a good example of this phenomenon (137;138). The transient decrease in insulin sensitivity during acute inflammation is potentially beneficial, as it allows the redistribution of glucose from one of its major consumers (skeletal muscle) to leukocytes. However, the decrease in skeletal muscle insulin sensitivity defines new set points of insulin production for pancreatic β-cells. These cells have the ability to increase insulin secretion to accommodate the new insulin sensitivity set point but can only maintain this over short periods. Therefore, if the inflammatory process is not resolved, the activity of pancreatic β-cells fails to compensate for the reduced skeletal muscle insulin sensitivity, and this may lead to

type 2 diabetes. The potential for adverse effects is intrinsic to any adaptive and persistent biological changes, regardless of whether these changes occur at the cellular, tissue or organism level.

1.3.3 An overview of biological pathways involved in inflammation

Although different cellular and molecular pathways characterize inflammation, all inflammatory processes can schematically be defined by the serial activation of four major components: inducers, sensors, mediators, and effectors (Figure 1.5).

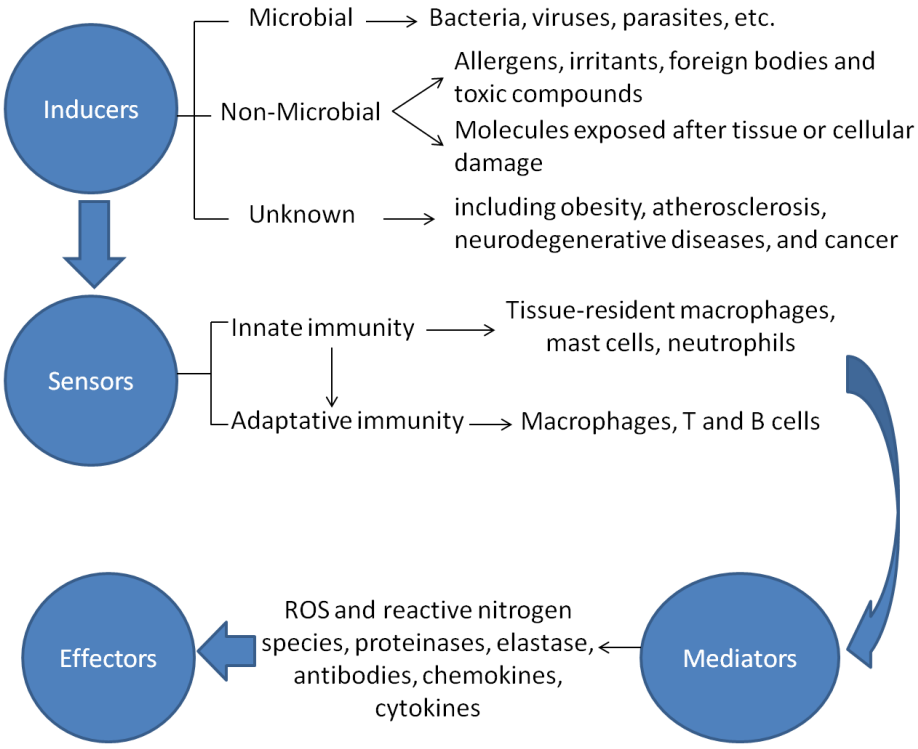


Figure 1.5 The inflammatory pathway.
A generic inflammatory pathway consists of inducers, sensors, mediators and effectors. (see text for further explanation).

Inducers are the inflammatory triggers that initiate the inflammatory response. They activate specialized sensors, which then elicit the production of a specific set of

mediators. The mediators then act on the effectors, the tissues and organs which are involved in the inflammatory process. The specific alterations induced in the effectors usually lead to the clinical manifestations of the inflammatory response. Indeed, the activity of the mediator determines specific changes in functions of tissues and organs.

Inflammatory inducers have a crucial role in influencing the cellular and molecular pathways involved in the inflammatory states. They can be generally grouped as microbial and non-microbial factors (figure 1.5). There are, in turn, two classes of non-microbial inducers: the first includes allergens, irritants, foreign bodies and toxic compounds, while the second includes molecules exposed after tissue or cellular damage. While this classification is relatively simple, it has been recently complicated by a growing number of acute and chronic systemic inflammatory conditions where the inflammatory inducer remains unknown (Figure 1.5). These states of systemic inflammation accompany many diseases of the western world, including obesity, atherosclerosis, neurodegenerative diseases, and cancer. It is currently thought that the evolution of such inflammatory processes depends on a vicious cycle connecting inflammation and the pathological processes they accompany. For instance, obesity can result from excessive food intake which causes adipose tissue dysfunction (accumulation of ectopic fat and adipose tissue inflammation), leading to a chronic inflammatory state (139). In turn, chronic inflammation can promote obesity-associated diabetes by contributing to insulin resistance (140), further increasing the baseline generation of oxidative stress and pro-inflammatory cytokines.

The activity of the inducers is recognised by specific cells which are either resident in the local tissue or recruited from the blood stream (sensors). These cells

are part of the innate and adaptive immunity, two systems developed to protect the human body from exogenous and endogenous aggression (141). The innate immune system is composed of cells (resident mast cells and macrophages as well as neutrophils) with rudimental defensive capacity, able to recognise potential inflammatory inducers and initiate the inflammatory response (142). However, the inflammatory response which follows activation of the innate immunity is not specific and targets not only the possible inflammatory triggers but also the surrounding tissues. If such a response is not able to remove the aggression by the inflammatory trigger, the release of several chemotactic molecules from the cells of the innate immune system activates more specialised cells which are part of the more sophisticated adaptive immune system (macrophages, B and T cells) (143). These cells produce cytokines, antibodies and other immune-inflammatory reactants which selectively target the inflammatory inducer, increasing the chance of a complete resolution of the inflammation and reducing the damage to surrounding tissues (144). Therefore, the interaction between innate and adaptive immunity orchestrates the evolution of the inflammatory response by production of multiple inflammatory mediators (141-143;145).

Inflammatory mediators can be classified into seven groups according to their biochemical properties: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines, proteolytic enzymes and antibodies (133;146). Each mediator will determine specific changes in local tissues (i.e. vasodilation, destruction of the connective tissue or cytotoxic activity on local cells and on the inflammatory trigger) which, if the aggression by the inducer or the inflammatory response is particularly severe, will result in the clinical manifestations of the inflammatory process.

1.3.4 Acute inflammatory responses

1.3.4.1 Cellular and molecular mechanisms involved in acute inflammatory responses

The cellular and molecular mechanisms involved in acute inflammation are highly dependent on the nature of the inflammatory inducers. Therefore, they have been treated separately on the basis of the specific inflammatory trigger.

1.3.4.1.1 *Microbial inducers*

Microbial inducers are usually recognised by the presence of pathogen-associated molecular patterns (PAMPs), a broad range of conserved molecular patterns commonly expressed on pathogens (whether pathogenic or commensal) but foreign to mammals (147). Examples of PAMPs include lipopolysaccharides (LPS), surface phosphatidylserine, and aldehyde-derived proteins, as well as modified forms of classical risk factors for atherosclerosis, including low-density lipoproteins (LDL) modified by oxidation or glycation. These molecules are detected by various scavenger and/or toll-like receptors (TLRs) of the innate immune system which are expressed on tissue-resident macrophages, neutrophils and mast cells (148).

Ligation of scavenger receptors can lead to endocytosis and lysosomal degradation of the bound ligands (149;150), while engagement of TLRs can lead to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades (151-155). Mediators amplify the inflammatory response, eliciting the production of a local inflammatory exudate. Plasma proteins and leukocytes (mainly neutrophils) normally restricted to the blood vessels gain access to the extravascular tissues at the site of infection.

The activated endothelium of the blood vessels at the inflamed site induces expression of surface selectins and integrins which interact with complementary ligands such as chemokine receptors on the leukocyte membrane allowing selective extravasation of neutrophils, while preventing the exit of erythrocytes (156). Upon reaching the affected tissue, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. Activated neutrophils release the toxic contents of their granules, including reactive oxygen species (ROS) and reactive nitrogen species (RNO), proteinase 3, cathepsin G and elastase in an attempt to remove the inflammatory stimulus (157). As these highly potent effectors do not discriminate between microbial and host targets, collateral damage to host tissues is unavoidable (158).

Although this sequence of events characterises most of the acute inflammatory processes evocated by microbial inducers, bacterial, viral, or parasitic factors can activate different sensors, mediators, and target tissues, such that the appropriate type of inflammatory response is induced. For example, viral infections induce the production of type-I interferons (IFN-α, IFN-β) by infected cells and the activation of cytotoxic lymphocytes (159), whereas infections with parasitic worms lead to the production of histamine, IL-4, IL-5, and IL-13 by mast cells and basophils (160).

1.3.4.1.2 *Non-microbial inducers*

The first class of non-microbial inflammatory triggers includes allergens, irritants, foreign bodies and toxic compounds (133). Allergens can be detected as they mimic the virulence activity of parasites, while irritants disturb the homeostasis and activate cells on epithelial surfaces. In both cases, the inflammatory response involves activation of mast cells and basophils because, similar to parasites, defence against

allergens and environmental irritants relies on expulsion and clearance mediated by the mucosal epithelia (161). Conversely, the inflammatory response evoked by foreign bodies is dominated by the phagocytic activity of macrophages (162).

Intra- and extra-cellular molecules that are normally kept sequestered in intact tissues can be released after acute tissue or cellular damage, representing the second important class of non-microbial inflammatory inducers. During necrotic cell death, for example, the integrity of the plasma membrane is disrupted, resulting in the release of certain cellular constituents, including ATP, HMGB1 (high-mobility group box 1 protein) and several members of the S100 calcium-binding protein family (S100A8, S100A9 and S100A12) (163;164). ATP binds to purinoceptors (including $P2X_7$) at the surface of tissue-resident macrophages, resulting in K^+ ion efflux, and can cooperate with other signals to activate the NALP3 inflammasome (165). Similarly, HMGB1 and S100A12 engage the receptor RAGE (advanced glycation end-product-specific receptor; also known as AGER), which (at least in the case of HMGB1) associates with TLRs to induce an inflammatory response (166;167).

Similarly, tissue injuries can cause damage to the epithelial cells, which normally separate the internal compartments from the external environment. This can cause “decompartementalization” and expose TLR receptors on macrophages, normally residing the lamina propria, which results in induction of a local inflammatory response. For example, damage to the vascular endothelium allows plasma proteins and platelets to migrate to extravascular spaces (156). A key plasma-derived regulator of inflammation, the Hageman factor (also known as factor XII), becomes activated by contact with collagen and other components of the extracellular matrix. Activated Hageman factor acts as a sensor of vascular damage and initiates the four proteolytic cascades that generate inflammatory mediators: the kallikrein–

kinin, coagulation, fibrinolytic and complement cascades (133). Platelets are also activated by contact with collagen and produce various inflammatory mediators, including thromboxanes and serotonin (133).

1.3.5 Inflammatory resolution: a road to chronic inflammation

Generally, inflammatory responses, evoked by tissue and cellular damage, exert beneficial effects as they promote tissue repair and help to prevent colonization of the damaged tissues by opportunistic pathogens. Their temporal evolution is restricted to the time necessary to eliminate the triggering insult and repair the local injury. Therefore, for effective resolution to occur, it is not only necessary to remove pro-inflammatory cells and molecules, but also to restore, as long as it is possible, the normal architecture and function of local tissues. Whilst termination of the inflammatory response is normally considered as a simple process which follows elimination of local pathogens, recent evidence demonstrated that this is a rather more complex process involving a number of highly regulated pathways (168). Cessation of proinflammatory signaling is a prerequisite that pre-empts removal of infiltrating granulocytes. During spontaneous resolution, neutrophils undergo apoptosis, a highly regulated cell death mechanism that prevents the release of histotoxic cellular contents (169). Alterations in neutrophil cell surface markers and morphological changes during apoptosis correlate with increased recognition by professional phagocytes, such as macrophages, that mediate effective clearance of dying cells (169;170). Once the pro-inflammatory environment has been removed, tissue restoration and return of tissue homeostasis become priorities. Successful post-inflammatory tissue repair requires the coordinated restitution of different cell types and structures, not only epithelial and mesenchymal cells but also extracellular matrix and vasculature.

Chemokines are critical to vascular remodelling after inflammation (171). Without appropriate restitution of the vasculature, altered tissue oxygenation may preclude normal repair, resulting in atrophy or fibrosis. Atrophy is often accompanied by expansion of extracellular tissue elements, particularly collagen, resulting in fibrosis and deposition of excess connective tissue. Fibrosis, sufficient to interfere with organ function, is a major medical problem after inflammation of arteries caused by accumulation of cholesterol, inflammation of the liver caused by viruses, alcohol, toxins or schistosome infections, inflammation of the lung associated with asthma or radiotherapy, and inflammation of the bowel in Crohn's disease, where fibrotic strictures often require surgery.

Persistent inflammatory stimuli or dysregulation of mechanisms of the resolution phase result in chronic inflammation (168), recognized to be a key underlying factor in the progression of a range of diseases, including atherosclerosis (172;173), arthritis (174), and chronic neurodegenerative diseases, such as Alzheimer's disease (175).

1.3.6 Chronic inflammatory responses

1.3.6.1 Cellular and molecular mechanisms involved in chronic inflammatory responses

Chronic inflammatory responses can be triggered by each of the inflammatory inducers causing acute inflammatory reactions. Similarly to acute inflammatory reactions, chronic inflammatory responses can have microbial and non-microbial origins and the cellular and molecular pathways involved in chronic inflammatory states are strongly influenced by the nature of the inflammatory trigger.

If microbial inducers are not efficiently removed during the acute inflammatory response, the neutrophil and macrophage infiltrate is progressively replaced by T cells (142). This shift from innate to adaptive immunity provides a more finely focused response mechanism that requires the recognition of specific molecular structures (antigens) on the surface of the inflammatory inducer (176). The induction of an adaptive immune response begins when a pathogen is ingested by an immature dendritic cell in the infected tissue (177). These specialized phagocytic cells are resident in most tissues and are relatively long-lived, turning over at a slow rate. They derive from the same bone marrow precursor as macrophages, and migrate from the bone marrow to their peripheral stations, where their role is to survey the local environment for pathogens. Eventually, all tissue-resident dendritic cells migrate through the lymph to the regional lymph nodes where they interact with recirculating naive lymphocytes (178). The immature dendritic cell carries receptors on its surface that recognize common features of many pathogens, such as bacterial cell wall proteoglycans (178). As with macrophages and neutrophils, binding of a bacterium to these receptors stimulates the dendritic cell to engulf the pathogen and degrade it intracellularly. The function of dendritic cells, however, is not primarily to destroy pathogens but instead to carry pathogen antigens to peripheral lymphoid organs, presenting them to T lymphocytes (178). When a dendritic cell takes up a pathogen in infected tissue, it becomes activated, and travels to a nearby lymph node. On activation, the dendritic cell matures into a highly effective antigen-presenting cell (APC) and undergoes changes that enable it to activate pathogen-specific lymphocytes that it encounters in the lymph node (179). Activated dendritic cells secrete cytokines that influence both innate and adaptive immune responses, making these cells essential gatekeepers that determine whether and how the immune system

responds to the presence of infectious agents (179). Presentation of microbial antigens to T-cells is followed by activation of cytotoxic T-cells (180) and production of antibodies by B-cells (181) that selectively target those molecules which evoke the immuno-inflammatory responses. T-cells can differentiate into at least two sub-phenotypes (known as T helper 1, Th1, and T helper 2, Th2) (182), which activate different patterns of inflammatory cytokines and control different limbs of the immuno-inflammatory processes (183) (Figure 1.6).

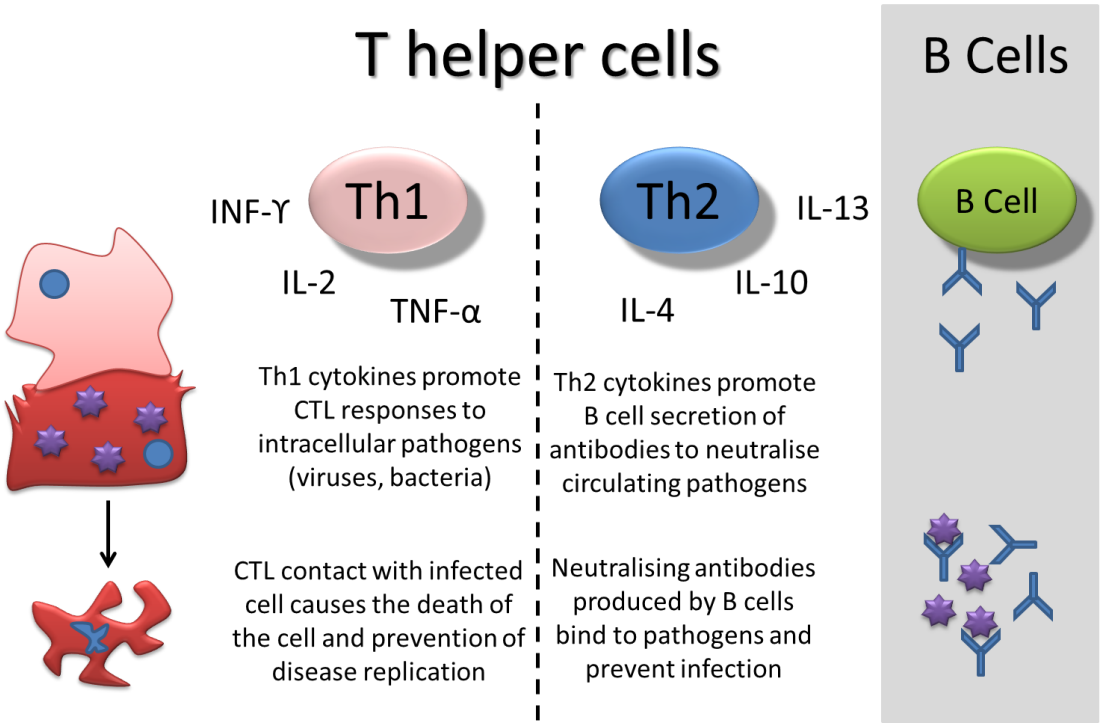


Figure 1.6 Role of Th1 and Th2 cells in the immune-inflammatory response
The adaptive immune response involves the generation of Th1 and Th2 cytokines. CTL: Cytotoxic T lymphocyte; Th: T helper.

Th1 cells produce IL-2, TNF-α, and IFN-γ, and are associated with cell-mediated immunity against intracellular pathogens (Figure 1.6), as well as being involved in delayed-type hypersensitivity skin reactions. Th1 cells also stimulate chemokines, which provide an important link between the recruitment of

inflammatory cells and adaptive immunity. Chemokines are pivotal in stimulating leukocyte migration from the blood to the tissues and can be secreted in response to Th1 cytokines, including IL-1 and TNF (184). By contrast, Th2 cytokines, such as IL-4, -5 and -10, are involved in the control of extracellular helminths infections and circulating pathogens by enhancing antibody-mediated immunity (Figure 1.6). However, they are also associated with allergic diseases including asthma, allergic rhinitis and eczema (atopic dermatitis) (184).

If the combined effect of macrophages and adaptive immunity is still insufficient to remove the inflammatory inducer, the persistent recruitment of inflammatory cells can lead to the formation of granulomas or tertiary lymphoid tissues (146;185). Formation of a granuloma follows the exposure of microbial (i.e. mycobacterium tuberculosis) and non-microbial (i.e. foreign bodies) inducers. Macrophages tend to aggregate around each other, forming a capsule of several cellular layers surrounding the pathogen (133;146). This process results in a sequestrum of the inflammatory inducer, neutralizing its ability to recruit and activate new inflammatory cells. Similar pathways of inflammation result from the activity of endogenous inflammatory triggers, such as crystals of monosodium urate and calcium pyrophosphate dihydrate (causing gout and pseudo-gout diseases, respectively) (186). Although the formation of a granuloma is effective in limiting the virulence of the inflammatory inducer, a state of chronic inflammation surrounding the pathogens can persist for decades inside this biological niche. The granuloma can reactivate at a later stage, causing a new acute inflammatory response. Globally, tuberculosis is the most prevalent example of chronic granuloma formation although similar processes have been described in other chronic disorders, including atherosclerotic plaques (187). When this inflammatory reaction is active enough to destroy the cellular walls,

products contained inside the granuloma are released in the surrounding tissues (i.e. lung), initiating another local and/or systemic inflammatory reaction.

During local chronic inflammatory responses, some triggers can elicit systemic and chronic inflammatory states. Examples of this include AGEs (advanced glycation end products) and oxidized low-density lipoproteins. AGEs result from the non-enzymatic glycation of long-lived proteins (i.e. collagen) (188) and can determine crosslinking of the proteins they are attached to, leading to gradual functional deterioration in these molecules. AGEs are recognized by specific receptors localized on the surface of mononuclear phagocytes and lymphocytes (RAGE), which have inflammatory activity either alone (167) or in combination with TLRs (189). Accumulation of AGEs has been described under hyperglycaemic and pro-oxidative conditions, including type 1 and type 2 diabetes, renal failure, neurodegenerative states and in general aging disorders (188). Similarly to AGEs, oxidized low-density lipoproteins can trigger systemic vascular inflammation by activating sub-endothelial or circulating macrophages (172). When these cells attempt to remove oxidated low-density lipoproteins from the sub-endothelial space using their scavenger receptors, they release potent oxidants, including the enzyme myeloperoxidase, that cause oxidation of other LDL-phospholipids and contribute to the chronic evolution of the inflammatory process (190).

1.3.7 Chronic inflammation as common link between aging, frailty and age-related diseases

1.3.7.1 Epidemiological evidence

Large population studies have repeatedly documented a 2–4-fold increase in serum levels of pro-inflammatory cytokines, such as IL-6 (also known as

"gerontologist cytokine") (191) and tumour necrosis factor alpha (TNF-α), with advancing age (191-195).

The most common explanation lies in the increased risk of chronic inflammatory diseases in the elderly which is likely to lead to increased concentrations of inflammatory markers in peripheral blood (193;196;197). However, 'successful aging' (aging without co-morbidity) is still associated with low-grade inflammatory activity *in vivo*. Numerous studies of older adults indicate that levels of several cytokines (including IL-6 and TNF-α) increase with age, even in apparently healthy individuals and in the absence of acute infection (191;192;198-200). These findings are confirmed by studies involving centenarians and frail people.

Centenarians are free of most age-related inflammatory diseases, despite their levels of circulating inflammatory markers being higher than those recorded in younger populations. Frailty is theoretically defined as a clinically recognizable state of increased vulnerability resulting from age-associated decline in reserve and function across multiple physiological systems, such that the ability to cope with everyday acute stressors is compromised (201-204). It can be considered an aging-plus syndrome where common co-morbidities recorded in the elderly are not present but their risk is significantly increased. In the absence of a gold standard, frailty has been operationally defined by Fried et al. as meeting three out of five phenotypic criteria indicating compromised energetics: low grip strength, low energy, slowed waking speed, low physical activity, and/or unintentional weight loss (9). Notably, each of these phenotypic criteria can be partially explained by increased levels of muscular and systemic inflammatory burden. The evidence that circulating inflammatory markers, such as IL-6 and its surrogate C-Reactive Protein (CRP), are strongly related to frailty syndrome, further reinforces the possible causal relationship

between levels of systemic inflammation and frailty (205). Additionally, recent reports have documented that not only IL-6 and CRP but also other but less specific inflammatory bio-markers including total leukocyte and neutrophil counts as well as albumin concentrations are associated with higher risk of frailty (14).

The association between aging, risk of age-related disease and increased levels of inflammatory markers raises an important question: what aspects of human biology underscore this association?

1.3.7.2 Possible biological pathways

1.3.7.2.1 *The central role of inflammatory cells*

The recent increase in human life expectancy presents novel challenges to the inflammatory system, which must now cope with chronic antigen exposure, lasting several decades beyond our evolutionary past. Decades of exposure to damaging agents results in a progressive remodelling process of the human body. Profound changes in the body's microenvironments through changes in protein abundance, composition and interaction are common findings during aging and represent continuous stimuli for the inflammatory system which must adapt its activities to establish new stable points for optimal bodily function (206). This continual antigenic stress leads to a progressive senescence of the immune-inflammatory system, characterized by accumulation of aged and activated immuno-inflammatory cells (207;208). As a result, a state of chronic low-grade inflammation develops, potentially contributing to the increased risk of age-associated frailty, morbidity, and mortality observed in the elderly. The attempt of the immune-inflammatory system to set new homeostatic set points to compensate for changes occurring over time in the human body leads to a process of immune-senescence, which is likely to explain the

increased level of circulating inflammatory markers (208). The accumulation of tissue stress and malfunction represent the immune-senescence inducers, with cells of the innate immune system, and particularly tissue resident macrophages, the main modulators of this process (207;209).

Tissue-resident macrophages constitute 10–15% of the total number of cells in most tissues, and their activity is crucial for maintenance of tissue homeostasis in basal conditions. Their functions extend beyond host defence as they adapt their level of activation, cytokine secretion and phagocytic activity to the local environment (210). Signals sent to macrophages from the surrounding tissue following stress differ from those sent in the basal state (211). For example, accumulation of dead or apoptotic cells in aged tissues leads to exposure of new antigens derived from modification or desequestration of intracellular proteins, lipids and nucleic acids. As a consequence, macrophages produce increased amounts and/or different sets of cytokines in the attempt to support tissue adaptation to the stressful conditions and restore its basal functionality. However, when stress or malfunction are extreme or irreversibly accumulate over time (such as during aging), the compensatory capacities provided by local macrophages are likely to be insufficient. Tissues might 'call for' the reinforcement of additional inflammatory cells, leading to a systemic inflammatory response and possible activation of adaptive immunity (212). The systemic inflammatory responses evoked by age-dependent tissue dysfunction are likely to be of lower magnitude than those due to infections or tissue injury and may explain the physiological increase of inflammatory markers observed during successful aging as well as during age-related diseases such as diabetes, cancer and atherosclerosis (208).

1.3.7.2.2 *The central role of oxidative stress*

The process of immune-senescence and the consequent (chronic) up-regulation of pro-inflammatory mediators (e.g., TNF-alpha, CRP, IL-6) during aging are currently considered to be primarily induced by an age-related redox imbalance that activates many pro-inflammatory signalling pathways (213). Maintenance of a precise redox balance is crucial for the optimal operation of homeostatic cellular activities. This status depends primarily on the balance between reactive species (RS), such as superoxide anions ($\cdot O_2^-$), hydroxyl radicals (·OH), nitric oxide (NO), peroxynitrite ($ONOO^-$), and anti-oxidant defence systems, such as superoxide dismutase (SOD), catalase, glutathione (GSH), and thioredoxin (Trx) (214). GSH is the most abundant intracellular thiol redox buffer and helps maintain redox status and protect cells against electrophilic oxidative attacks (215). Mitochondria represent, by contrast, the primary source of oxidative factors in physiological conditions, as mitochondrial respiration is normally coupled with production of high amount of oxidative by-products (216). During aging, anti-oxidant defence systems (such as intracellular GSH levels) generally decline (217), while generation of RS (especially of mitochondrial origin) progressively increase (218). The up-regulation of the RS bioavailability observed during aging ultimately leads to higher levels of inflammation, by either direct or indirect mechanisms.

1.3.7.2.2.1 <u>Indirect mechanisms of RS-induced inflammation</u>

The indirect mechanisms by which RS can cause an increased inflammatory burden lies in their ability to modify the function of the three main classes of cellular macromolecules (lipids, nucleic acids, and proteins)(Figure 1.7).

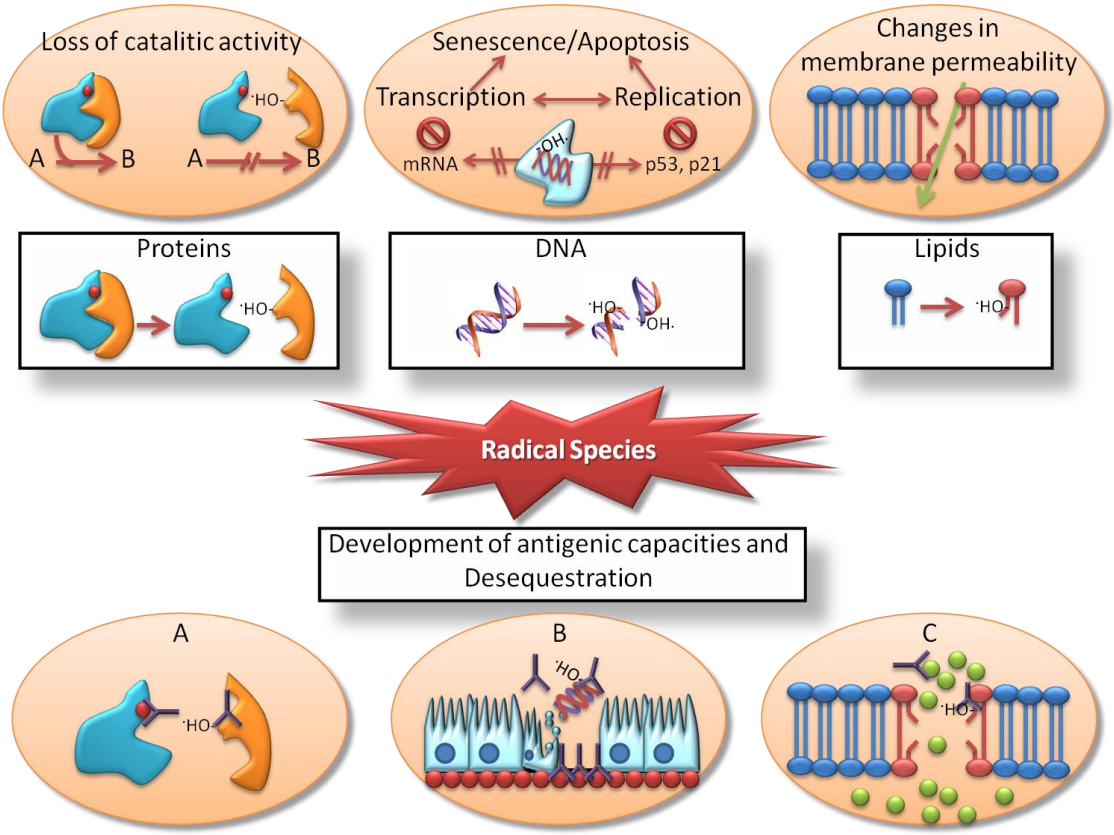


Figure 1.7 Oxidative mediated cellular damage.
Each intracellular macromolecule can be damaged by increased bioavailability of oxygen radical species. Oxidative damage to: A) proteins leads loss of catalytic activity, B) nucleic acids leads to activation of the DNA damage response or modification of mRNA, causing arrest of cell cycle and cellular senescence or apoptosis, C) lipids leads to increase membrane permeability and loss of the trans-membrane homeostasis. These processes can lead to activation of the immune system, either because structural changes make macromolecules recognised as foreign bodies by the immune systems or because of decompartmentalization and exposure of antigens that normally are sequestered inside cells.

This can lead to tissue malfunction and associated up regulation of the inflammatory response by several mechanisms:

1. RS can react with cell membrane fatty acids and form lipid peroxides, resulting in permanently impaired fluidity and elasticity of the membrane, and consequent cell rupture (219) (Figure 1.7). Similarly, overproduced radicals can react with protein amino acids leading to their oxidation and cross-linking. Radical-protein reactions can permanently impair the function of important

cellular and extracellular proteins, including enzymes and connective tissue proteins (Figure 1.7). It has been estimated that oxidized protein in old rats may comprise 30-50% of the total cellular protein (220). DNA is another macromolecule which is highly susceptible to free radical attack. An oxygen radical interaction with DNA can break its strands or delete a base. This DNA damage can be a lethal event for an organism, as it can cause an irreversible arrest of cell replication (Figure 1.7). It has been estimated that, on average, more than 10,000 oxidative hits occur each day in the DNA of a single human cell (221).

2. Structural changes induced by free radical damage have the potential to transform inert macromolecules into potent inflammatory inducers. One example is the conversion of inactive low density lipoproteins (LDL) into their highly pro-inflammatory counterparts (ox-LDL), a process determined by oxidation of the lipid and protein components of the lipoproteins (172).
3. Functional changes induced by free radicals in membranes, proteins and nucleic acids lead to progressive cellular and tissue damage, followed by desequestration of endogenous antigens (Figure 1.7). For instance, increased exposure to oxidative stress seriously damages endothelial cells which not only become dysfunctional, but also lose integrity, progress to senescence, and detach into the circulation (156). As previously discussed, sites of "disendothelization" represent potent activators of the Hageman factor, which interacts with components of the extracellular matrix and initiates the inflammatory response by stimulating the kallikrein-kinin, coagulation, fibrinolytic and complement cascades (133). Oxidative damage to intracellular components has been suggested not only as the primary source of

inflammation during aging but also as the most important pathway accounting for the aging process.

The “Free Radical Theory of Aging” proposed in the 1950s by Denham Harman (222), postulates that oxygen free radicals, formed endogenously from normal metabolic processes, play a central role in the aging process because of an increase in oxidative damage to macromolecules. This theory has since been modified to the “Oxidative Stress Theory of Aging” because oxygen species such as peroxides and aldehydes, which are not technically free radicals, also play a role in oxidative damage to cells. According to the former theory, the imbalance between pro-oxidants and antioxidants leads to oxidative damage in a variety of macromolecules with age, resulting in a progressive loss in functional cellular processes and to the emergence of aging phenotypes (223).

1.3.7.2.2.2 Direct mechanisms of RS-induced inflammation

Over the last few years, it has become clear that an increased bioavailability of RS can induce chronic inflammatory responses by direct mechanisms. Several studies demonstrated that, the pro-inflammatory effects elicited by oxidative damage to cellular macromolecules, gene expression of pro-inflammatory peptides such as IL-1β, IL-6, TNF-α, cyclo-oxygenase-2 (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS) are enhanced during aging by the redox-sensitive transcription factor nuclear factor-κB (NF-κB) (224-226). Similarly, current research suggests a central role for RS of mitochondrial origin (mtRS) in mediating the increased inflammatory levels observed in the elderly (218). While mitochondria are crucial for normal cell functions, their respiratory activity is coupled with the release

of high levels of RS which, in turn, act as signaling molecules, directly triggering pro-inflammatory cytokine production (227-229).

In order to prevent excessive accumulation of mtRS, cells developed a complex mechanism, known as mitophagy (more generally known as “autophagy”), to control the number of mitochondria within a cell by removing those which become dysfunctional and which produce a high amount of mtRS (230). Autophagy is an essential cyto-protective pathway and consists of the formation of autophagosomes, double-membrane vesicles that sequester organelles, proteins, or portions of the cytoplasm, which then fuse with lysosomes (231). As a result, the sequestered contents are degraded by lysosomal enzymes, and recycled as a source of energy (231). Autophagy may occur either as a general phenomenon, for instance when cells lack nutrients and mobilize their energy reserves, or it can specifically target distinct cellular structures such as damaged mitochondria (“mitophagy”) (232).

Inhibition of autophagy results in the accumulation of damaged mitochondria in human cells, leading to an increase in the net amount of mtRS and pro-inflammatory cytokine production (229). Interestingly, autophagy appears to decline with age, and gene expression of key regulators in the autophagic pathway (i.e ATG5 and ATG7) are reduced in aging individuals (233). Additionally, major human age-related diseases characterized by an increased inflammatory burden (i.e. Parkinson’s and Alzheimer’s disease) have been linked to defects in mitochondrial autophagy (234-236). These data strongly support a possible contribution of mtRS to the age-dependent increase of the inflammatory burden. Interestingly, conditions that promote autophagy, such as caloric restriction and exercise, delay aging-associated degeneration (237), suggesting that autophagy exerts important roles also in controlling the evolution of aging. Stimulation of autophagy can increase the healthy

lifespan in multiple model organisms including mice and primates (238), while experimental inactivation of genes required for the execution of autophagy is lethal at the whole-body level, whereas tissue-specific knockouts induce organ-specific degenerative changes (239).

1.4 LEUKOCYTE TELOMERE LENGTH AND INFLAMMATION

Both epidemiological and experimental studies suggest that inflammation, aging and age-related diseases are intimately related with each other and have proposed oxidative stress as the main link between them. However the potential molecular pathways accounting for these associations are yet to be defined.

Ideally, this pathway but should be:

1. Influenced by cumulative inflammatory and oxidative stress exposure,
2. Epidemiologically related to the risk of morbidity/mortality for age-related diseases and
3. Biologically involved in the mechanisms of cellular aging.

Telomere length and its biology could easily account for the strong link between aging and inflammation, as it is centrally involved in the mechanisms of cellular aging, relates with the risk of morbidity/mortality for many age-related diseases and can potentially be affected by oxidative stress damage. However, the potential impact of long term inflammatory and oxidative stress exposure on telomere length has not been explored in humans.

1.4.1 Potential pathways linking LTL with chronic inflammatory and oxidative stress exposure

Prolonged or extreme conditions of tissue stress or malfunction (related to exogenous or endogenous aggressions) induce production of pro-inflammatory cytokines by tissue resident macrophages (210). These cytokines (directly or indirectly) stimulate proliferation and differentiation of new inflammatory cells (leukocytes) from the bone marrow, increasing leukocyte count in the peripheral circulation. The increased concentration of circulating leukocytes provides adaptive

benefits, as it increases the number of inflammatory cells available to restore local homeostasis. Once recruited to the site of injury, many of these cells however die, releasing their intracellular content in the surrounding tissues. Products of damaged, apoptotic and necrotic cells further amplify the inflammatory process, as they stimulate production of pro-inflammatory cytokines by tissue-resident macrophages (133). These inflammatory mediators circulate from the injured tissue to the bone marrow, increasing the rate of HSC replication and telomere shortening (240). As leukocytes originate from HSC, an increased rate of telomere attrition in these cells will be reflected in shorter telomere length measured in peripheral leukocytes (113). This pathophysiological response to tissue stress and/or malfunction is thought to account for the LTL shortening observed not only during inflammatory states, but also during the physiological evolution of aging.

The increase in systemic oxidative stress, commonly associated with acute and chronic inflammatory states, is another factor which may increase telomere shortening in HSCs and peripheral leukocytes (42). There are two main mechanisms by which oxidative stress is presumed to accelerate LTL shortening (241):

1. Through increasing the number of damaged telomere sequences and reducing the activity of telomerase in HSC.
2. Through damaging genomic DNA which in turn leads to telomere independent senescence and apoptosis, diminishing the biological life of peripheral leukocytes. This enhances HSC replication to accommodate peripheral needs, indirectly shortening LTL.

Although increased inflammation is commonly considered the cause of LTL shortening, recent evidence suggests that reverse causality and residual confounding might account for the relationship between LTL and inflammation. Indeed, the

persistent activation of the DNA damage response, due to critically shorter telomere length, activates production of pro-inflammatory cytokines (i.e. IL-6) in cultured human fibroblast (242). In this context, cellular aging reflected by shortened LTL represents a cause and not consequence of inflammation.

1.4.2 Epidemiological evidence linking inflammation with LTL

Whilst a number of observational studies (mainly cross-sectional) have explored the association between inflammatory biomarkers and LTL, evidence remains inconclusive. In women, Fitzpatrick et al., failed to find any association between levels of CRP and LTL in elderly individuals (63). In contrast, Richards et al reported an association between CRP and LTL in a population of 1207 middle-aged females (243). This association was partially confirmed by Aviv et al. who demonstrated that CRP is inversely correlated with LTL in premenopausal but not postmenopausal women (65). Regardless of the possible gender interaction, Bekeart et al demonstrated a negative association of CRP and IL-6 with LTL in middle aged male and female subjects free of overt CVD (75). Similar results were later reported by O'Donovan et al in an elderly population (aged 73 years) (244).

Several studies, however, failed to find any association between inflammatory biomarkers and LTL. Indeed, in a population of 2744 elderly men included in the Osteoporotic Fractures in Men-Sweden study, Moverare-Skrtic and colleagues found no association between CRP and LTL (245). Similarly, Brouilette et al. could not find any association between LTL and circulating levels of CRP and fibrinogen in a sub-analysis of the West of Scotland Primary Prevention Study, including 1544 males and female subjects aged 56 years (61).

1.4.3 Epidemiological evidence linking oxidative stress with LTL

The association between LTL and parameters of oxidative stress exposure is one of a complex nature. Although the influence on telomere length of intra and extracellular levels of superoxide dismutase (246), glutathione dependent redox homeostasis, hydrogen peroxide and Cu(II), as well as Thiol-specific anti-oxidant 1 (Tsa1) has been reported thus far in living yeast (247) and cultured cells (48;246;248), the extent to which these findings can be translated into humans remains unclear. Indeed, only a few studies have analysed the relationship between oxidative stress and LTL in large populations.

Epel et al. found that psychological stress was significantly associated with increased oxidative stress, determined by F2-isoprostanes (F2-IsoP, a class of major oxidative by-products) levels quantified from a 12-h nocturnal urine sample (249). The study was performed in 58 premenopausal women who were biological mothers either of a healthy or a chronically ill child, the latter been a predictor of greater environmental exposure to stress. Lower telomerase activity and shorter telomeres were detected in the group with greater exposure to stress. The groups were matched with respect to age, smoking behaviour, and vitamin use, but the high-stress group had a significantly higher body mass index (249). Whilst this study suggests a role for oxidative stress in telomere length regulation in humans, the authors were unable to describe a direct association between levels of oxidative stress biomarkers and telomere length.

In other studies, circulating levels or diet intake of antioxidant compounds (such as vitamins and minerals) have been used as possible surrogate markers of oxidative stress exposure. In a population of middle-aged people with type 2 diabetes, Salpea et al reported that a cross-sectional measure of plasma total antioxidant status

(TAOS) was associated in a linear fashion with LTL (250). Interestingly, within the same population, individuals carriers of the common functional variant 866G>A in the promoter of the human uncoupling protein 2 (UCP2) gene (which results in a reduced clearance of mtRS by UCP2 and is associated to reduced levels of TAOS) had shorter age-adjusted LTL when compared to common homozygous 866GG, suggesting a possible role for mitochondrial oxidative stress production in LTL regulation (250). On the other hand, Richards et al found serum vitamin D concentrations were positively associated with LTL (251). This association was observed in a population of 2160 women aged 18–79 y (mean age: 49.4) and persisted after multiple adjustment for age, season of vitamin D measurement, menopausal status, use of hormone replacement therapy, and physical activity. Interestingly, the direct relationship between short LTL and low vitamin D levels was stronger in subjects with increased concentrations of CRP (251). The same group subsequently reported a negative association between LTL and plasma homocysteine levels in 1,319 healthy subjects recruited from the same population (243). This association persisted after adjustment for smoking, obesity, physical activity, menopause, hormone replacement therapy use and creatinine clearance. Finally, Xu et al described longer LTL in participants of the Sister Study who consumed higher levels of vitamins (particularly vitamin C and D) compared to the rest of the population (252).

All these studies provide indirect evidence for a possible role of oxidative stress in the regulation of LTL. Only one report to date showed a direct association between circulating levels of oxidative stress and LTL in humans. In this case-control study, Wolkowitz et al showed that LTL of 18 not medicated patients with major depressive disorder were shorter than 17 healthy controls (253). The authors described

a negative association between LTL and circulating levels of oxidative stress, as assessed by the ratio between F2-IsoP and the anti-oxidant vitamin C.

1.4.4 Difficulties in exploring the association between LTL, inflammation and oxidative stress

Exploring the association between LTL, inflammation and oxidative stress is further complicated by the different kinetics between LTL shortening and change of inflammatory markers. Whilst biomarkers of inflammation and oxidative stress presumably reflect the metabolic status at the moment of sample collection, LTL represents the cumulative burden of inflammation and oxidative stress over the individual's life span (75;240;241). This difference may be critical in both healthy and disease populations. In healthy individuals, acute infections at the time of blood collection can determine an "artificial" increase of inflammatory markers such as CRP, IL-6 and fibrinogen. These inflammatory responses, however, are unlikely to impact on LTL dynamics, which requires prolonged exposure periods before damage can be detected. Similarly, in diseased populations (i.e. subjects with diabetes, periodontitis, rheumatoid arthritis or CVD), circulating levels of inflammatory markers are likely to be affected by fluctuations in the activity of the underlying disease and may not reflect the level of chronic inflammatory burden (254-262). A better estimation of inflammatory impact on LTL in humans would therefore require identification of novel inflammatory markers, providing information on the chronic rather than the acute inflammatory burdens.

Another source of variability relates to the methods used to measure LTL and levels of circulating inflammatory markers. Highly sensitive biochemical tests for CRP and IL-6 quantification can detect very small differences in circulating levels of

these inflammatory cytokines. In contrast, laboratory techniques used to measure LTL are unable to detect such small differences (80;263). This problem can be further complicated in healthy populations. For example, one could decide to perform an analysis excluding individuals with abnormally high levels of CRP (i.e. >3mg/L) from a study cohort, assuming that these levels reflect the presence of the ongoing acute inflammatory response. The remaining population will be composed of individuals with mild differences in chronic inflammatory burden which presumably is reflected in a relatively small inter-individual difference in LTL. In this case, the LTL assays would not have sufficient sensitivity to capture such a small inter-individual difference. The reproducibility and sensitivity of the LTL assay is therefore the limiting factor for accurate and reliable analysis.

Conversely, the lack of stable and reliable markers of oxidative stress limits our ability to explore an association between LTL and oxidative stress. Following their generation, free radicals react with surrounding macromolecules, making their half-life extremely short. Thus, direct measures of reactive oxygen species in peripheral blood are difficult to perform and often require the use of very expensive machines (e.g. electron paramagnetic resonance) and adoption of complicated protocols (264;265). Such characteristics limit their application and utility in large epidemiological studies.

Researchers have therefore used alternative approaches to estimate levels of oxidative stress exposure, commonly based on the measure of the amount of structural damage to macromolecules resulting from interaction with oxidative species (266) (Figure 1.8).

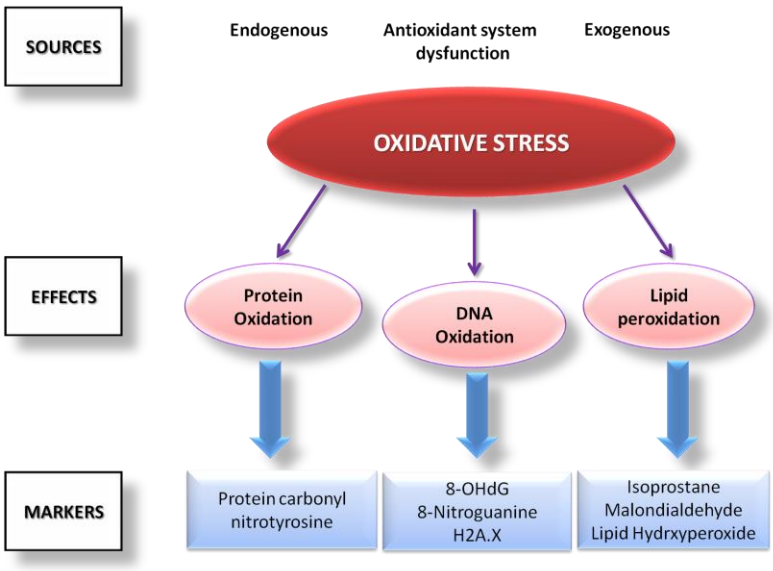


Figure 1.8 Markers of oxidative stress derived from RS damage to macromolecules. *Free radicals have a short half-life as they quickly interact with surrounding macromolecules, forming more stable compounds. Therefore, by-products derived from these interactions have been used as surrogate measures of total oxidative stress exposure.*

Several stable by-products of lipid, protein and DNA oxidation have been identified in biological samples, making the assessment of the oxidative stress exposure easier and less variable. Examples include:

1. Serum lipid hydroperoxides (LOOH), generated from polyunsaturated fatty acids and representing primarily products of fatty acid peroxidation (267).
2. Malondialdehyde (MDA), the by-product of the arachidonate cycle and a principle aldehyde product of lipid peroxidation *in vivo* (268).
3. F2-IsoP, formed by the free radical catalysed peroxidation of phospholipid bound arachidonic acid (269).

LOOH and MDA have been found to be elevated in chronic inflammatory diseases (e.g. diabetes) as well as with pro-oxidant behaviours (e.g. cigarette smoking) (270;271). In addition, urinary and plasma levels of F2-IsoP have been shown to correlate with pro-oxidant risk factors (272). Despite this evidence, stability and the

sensitivity of these markers has not been tested in humans and it remains unclear whether a single measure of lipid peroxidation can accurately reflect the total oxidative stress burden of the individual. Similar limitations can be described for by-products of protein and DNA oxidation, making the interpretation of associations between oxidative stress and LTL difficult. As a result, it has been suggested that assays able to detect the total oxidative capacity of blood may be more informative than individual oxidative stress measures.

1.5 CHRONIC INFLAMMATORY DISEASES: A METHOD TO STUDY THE EFFECTS OF CHRONIC INFLAMMATION IN HUMANS

It is now recognized that a mild pro-inflammatory state is correlated with the major degenerative diseases of the elderly. This inflammatory state has been commonly measured by a range of biomarkers (such as CRP or IL-6) which reflect current levels of inflammation. However, these markers are unable to provide information on the individual's inflammatory burden. As chronic inflammation is a pathological condition characterized by a continued active inflammatory response leading tissue destruction, and measures of tissue remodelling are more likely to reflect the cumulative inflammatory burden than levels of circulating inflammatory markers.

In many cases, tissue remodelling is not only a consequence of chronic inflammation but also a marker of disease evolution. For example, the chronic inflammatory process involving the CV system during atherosclerosis determines a progressive thickening of the arterial wall, which can lead critical ischaemia and infarcts. Although several risk factors can contribute to the process of vascular

remodelling, it is now clear that these factors do not completely explain the evolution of CVD. Indeed, vascular remodelling can be detected in old individuals without a significant CV risk factor burden.

In this thesis, periodontitis (PD) and CVD were used as models of systemic and chronic inflammatory burden to explore the relationship between measures of tissue remodelling and LTL. Furthermore, the association between LTL dynamic and vascular phenotypes was explored in a cohort of individuals with low CV risk factor burden in order to define the relationship between dynamic regulation of LTL and evolution of age-related disease.

PD and CVD were used as: a) they are common in the general population, b) they share common mechanisms/pathophysiology with other age-related diseases such as cancer and type 2 diabetes, c) the degree of tissue remodelling is easy to quantify using non-invasive techniques and d) the amount of tissue remodelling is correlated with levels of chronic inflammation and relates to outcome.

1.5.1 Periodontitis: a model of chronic and systemic inflammation associated with accelerated aging

Periodontal diseases, including gingivitis and PD, are amongst the most commonly occurring chronic infections in humans, due to the anatomically unique periodontal structure and the nature of the pathogenic dental plaque biofilm infection (273;274). PD is characterized by bacteria-induced inflammatory destruction of tooth-supporting tissues including alveolar bone, and it remains a major cause of tooth loss in adults in both developed and developing countries (275).

In susceptible individuals, the disease is initiated by specific Gram-negative microflora which appears organized on the tooth root surfaces as a dental biofilm (276-278). The interaction between bacteria and local inflammatory cells results in the production of pro-inflammatory cytokines and chemokines, which recruit additional polymorphonuclear cells, monocytes/macrophages, T and B cells to the site (gingiva) (279-282). While host inflammation is normally able to remove the inflammatory inducers and restore local tissue homeostasis, in susceptible individuals, the bacterial aggression is not effectively counteracted by the host due to a dysregulation of the immune-inflammatory response (282;283). This leads to the formation of inflammatory infiltrates in the connective tissue adjacent to the gingival pocket epithelium (Figure 3.1).

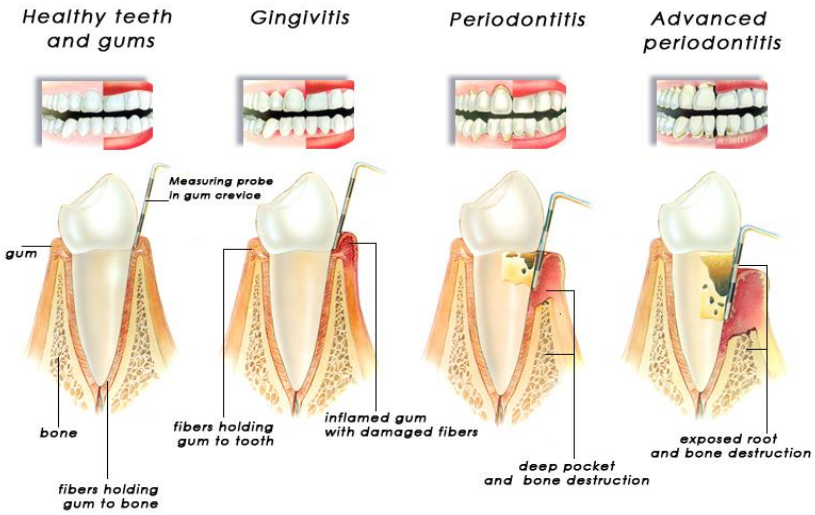


Figure 1.9 Progressive evolution of periodontal inflammation.
Periodontitis is an oral infection caused by accumulation of a bacteria-containing biofilm around the teeth. As this biofilm matures, more virulent bacteria cause an inflammatory reaction in the soft and hard tissues supporting the teeth. If the biofilm is not removed periodically, the initial inflammation of the gingiva (referred to as gingivitis) may develop into a more serious long-term destructive process (periodontitis) in which the periodontal ligament and bone supporting the teeth are progressively eroded. Left untreated, this may result in bleeding around the teeth, abscess formation, gingival recession, loosening or complete loss of teeth (advanced periodontitis).

The cytolytic activities of granulocyte-derived enzymes as well as the cellular damage due to the increased levels of oxidative stress lead to a progressive destruction of the surrounding tissues, including the alveolar bone (284-287). This has a progressive impact, beginning with gingival recession and progressing toward tooth mobility, pathological migration, and, eventually, tooth loss (288). Retraction of gingival tissue and alveolar bone loss may be considered as clinical markers reflecting the amount of inflammatory periodontal exposure.

Although there is not universal agreement in the definition of different forms of PD, the disease is commonly divided into two major variants: chronic and aggressive PD (289). Chronic PD marks a constellation of destructive periodontal diseases characterized by a long evolution of a moderate inflammatory process, which is usually diagnosed in adult age. In contrast, the aggressive form is highly destructive, usually diagnosed in young age and characterized by an inconclusive medical history, familial aggregation and a higher local and systemic inflammatory involvement (289). The highly active inflammatory process leads to the appearance of early clinical manifestations, reducing the time to the diagnosis and providing a higher chance for an effective treatment.

It is now well established that PD affects not only oral health, but also results in a systemic inflammatory response, possibly due to entry of bacteria in the blood stream which cause activation of the host inflammatory response by multiple mechanisms. (290-293).

Over the past 8 years, our group has undertaken an extensive characterization of the links between severity of the oral disease and levels of systemic inflammatory markers. We have shown that individuals with PD not only present increased levels of

CRP and IL-6 in peripheral blood, but also that these levels change accordingly with the evolution of the oral disease (254;255;294) (Figure 3.2).

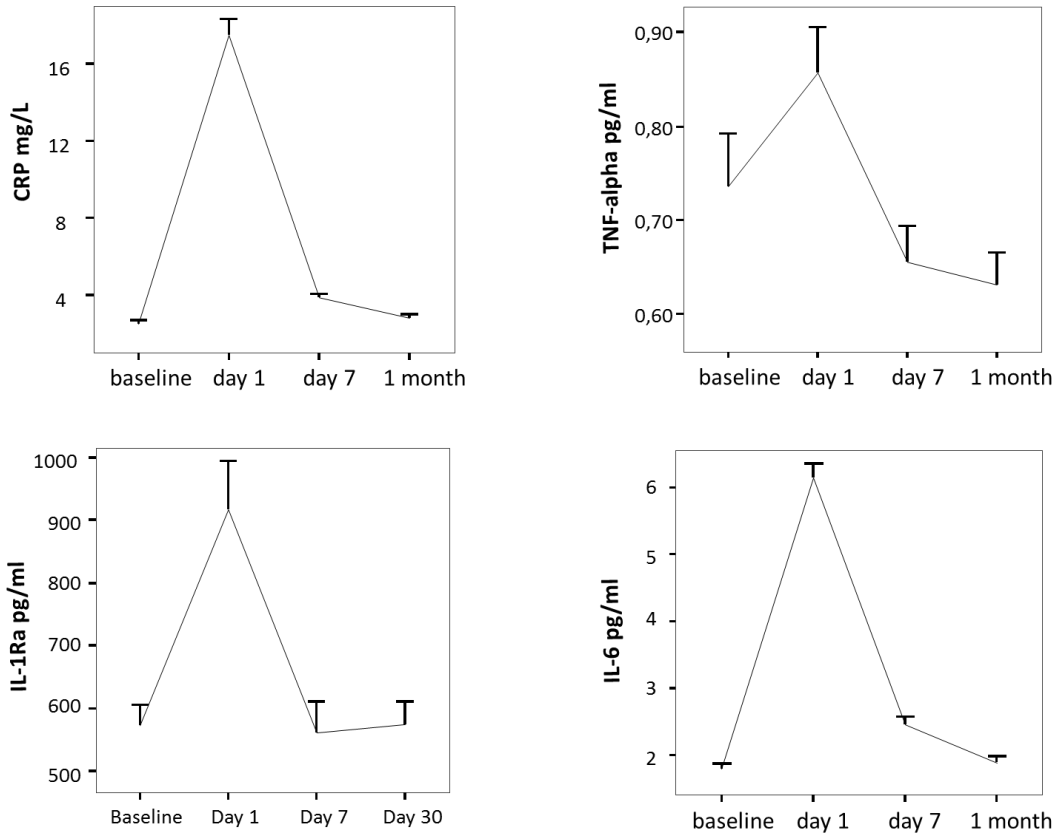


Figure 1.10 Changes of systemic inflammatory markers following intensive periodontal treatment
Periodontal treatment is associated with acute changes in levels of systemic inflammatory markers. An acute increase of CRP, TNF-α, Interleukin-1 Receptor (IL-1Ra) and IL-6 occurs immediately following dental treatment with a peak at 24 hours. The inflammatory markers recover one week following treatment and return to baseline values after one month.

Furthermore, we have reported that changes in systemic inflammatory markers are associated with changes in systemic oxidative stress exposure (295). This data underscores the strong link between oral and systemic inflammatory involvement in individuals with PD, possibly explaining their increased risk of morbidity for age-related systemic disorders. Indeed, PD has been recognised as a possible risk factor for several age-related diseases (275;296-298), including CVD (299;300), diabetes

(301-304), cancer (305;306), cognitive decline (307;308), renal failure (309-312), chronic respiratory diseases (313-316) and osteoporosis (317-320).

Whilst systemic inflammation is likely to represent the missing link relating PD with a higher risk of mortality and age-related disease, the underlying biological pathways remain largely unexplored. Within the context of this PhD, PD represented a unique and useful model of local and systemic inflammatory disease and accelerated aging to explore the link between LTL, levels of oxidative stress and chronic inflammation.

1.5.2 Atherosclerosis: an age-related disease led by inflammation

CVD is the most frequent age-related disease in developed countries, followed by cancer. The most frequent cause of CVD is atherosclerosis, an inflammatory process of the vascular wall which determines focal thickenings of the innermost layer of the artery, the intima (321)(Figure 3.3).

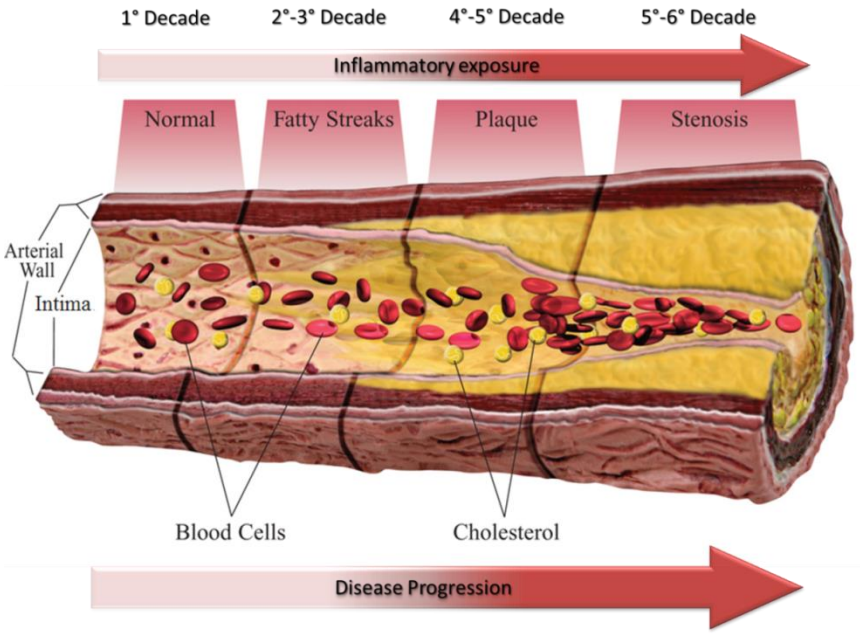


Figure 1.11 Evolution of atherosclerosis with age and duration of inflammation. *Atherosclerosis is an inflammatory disease of the vasculature, characterised by accumulation of cholesterol in the innermost layer of the vascular wall, the intima. The progressive narrowing of the vessel due to lipid accumulation progressively reduce the blood supply to peripheral organs, leading to clinical manifestations of the disease, which normally become evident at 50-60 years old.*

The pathophysiological evolution of atherosclerosis is characterized by accumulation and retention of LDL cholesterol in the intima, particularly at sites of hemodynamic strain (322). Oxidative and enzymatic modifications of LDL particles lead to the release of inflammatory lipids that induce endothelial cell activation (323), characterized by reduced bioavailability of nitric oxide, increased production of oxidative metabolites and expression of leukocyte adhesion molecules (324). Monocytes recruited through the activated endothelium differentiate into macrophages (325). Several endogenous and microbial molecules can ligate pattern-recognition receptors (TLR) on these cells, inducing activation and leading to the release of inflammatory cytokines, chemokines, oxygen and nitrogen radicals, and other inflammatory molecules, ultimately amplifying the local and systemic inflammation/oxidative stress levels and causing tissue damage (326-328).

Figure 1.12 Biological pathways in the initiation of atherosclerosis.
Excess LDL infiltrates and is retained in the sub-intima space at sites of hemodynamic strain. Oxidation of LDL leads to endothelial cell activation which mediates migration of inflammatory cells (mainly monocytes) in the sub-intima space, where they become activated, mature to macrophages and express TLRs and scavenger receptors. Adapted from Andersson et al.Clin Immunol 2010;134(1):33-46.

These early atherosclerotic lesions (known as fatty streaks) evolve with the accumulation of both intracellular and extracellular lipid and debris, which further activate inflammatory cells and contribute to oxidation of new LDL molecules (329;330). These newly formed oxLDL molecules increase recruitment and activation of other inflammatory cells, damage local endothelial and smooth muscle cells, thus creating a cycle which actively contributes towards progression of the local inflammatory process and lesions (331). Focal inflammatory responses acquire a more organised structure, with lipids and debris accumulating in a necrotic core surrounded by a fibrous cap, composed predominately of vascular smooth muscle cells and their secreted products (collagen and elastin) (332)(Figure 3.5).

Figure 1.13 Evolution of atherosclerosis and organization of atheroma.
The atheroma has a core of lipids, including cholesterol crystals, living and apoptotic cells and a fibrous cap with smooth muscle cells, collagen and elastin. Plasma lipoproteins accumulate in the central region of plaques (core). Several types of cells of the immune response are present throughout the atheroma including macrophages, T cells, mast cells and dendritic cells (DCs). The atheroma builds up in the intima-media layer of the artery, progressively narrowing the arterial lumen by increasing the thickness of the vascular wall. Adapted from Hansson et al. Nature Immunology 2011;12:204-212.

All these steps cause a further increase in intima-media thickness, with formation of atherosclerotic plaques. Therefore, inflammatory cells and their reactive oxidant species represent crucial components in both the initiation and evolution of atherosclerosis.

A decade ago, the treatment of common CV risk factors and the introduction of lipid lowering therapies were expected to prevent evolution of vascular remodelling which is involved in atherosclerosis. Lately, however, the increasing effectiveness of CV treatments has demonstrated that structural changes of the vascular wall can be detected also in individuals with optimal levels of CV risk factors, suggesting that intimal and medial thickenings as well as gradual loss of arterial elasticity may represent, at least in part, physiological processes observed during aging (333). It is now clear that aged vessels share a number of structural modifications with atherosclerosis (334-336). For example, aged vessels show fracture of the elastin lamellae and increased collagen deposition, which may lead to vessel dilation as well as increased lumen size and intima-media thickness (337). Furthermore, the increased collagen and decreased elastin content of the vascular wall, promoted at least in part by age-associated increases in glycated proteins, matrix metalloproteinase enzyme activity, and trophic stimuli such as angiotensin II signaling, impair vessel elasticity and hence promote vascular stiffness (338). These alterations are commonly found also in earliest phases of atherosclerosis (332).

The strong connection between aging and atherosclerosis is confirmed by the evidence that a number of genetic diseases associated with premature vascular aging are commonly associated with CV complications including atherosclerosis. For example, Hutchinson Gilford progeria syndrome (HGPS) is a rare, fatal, and

progressive premature aging condition and young patients reveal accumulated collagen, fractured elastin lamellae, and a thickened intima, with some vessels showing advanced atherosclerotic lesions containing chronic inflammation, calcification, and vascular smooth muscle cell loss (339). Another example is the Werner syndrome (WS), a loss-of-function mutation in the WS ATP-dependent helicase (WRN), which shows a similar pathology and accelerated atherosclerosis (340-342).

In addition to the evidence provided by rare genetic syndromes, there are strong connections between cellular aging pathways and atherosclerosis. There is increasing evidence that vascular smooth muscle and inflammatory cells within atherosclerotic plaques have accumulated DNA damage, and that vascular smooth muscle cells of atherosclerotic plaques undergo the consequences of DNA damage, including apoptosis and premature senescence (343). DNA strand breaks and chromosomal damage are present in circulating cells of patients with atherosclerosis; DNA damage correlates with a higher micronucleus index (a marker of genetic instability often recorded in aging cells) compared with healthy controls, and it is associated with disease severity (344). Vascular smooth muscle cells and macrophages express markers of DNA damage in plaques, including phosphorylated forms of the Ataxia Telangiectasia Mutated (ATM) and Histone 2A protein X proteins (γ-H2A.X) (345). Importantly, these markers increase with disease severity. *In vitro*, increased DNA damage can be observed in vascular smooth muscle cells derived from atherosclerotic lesions compared to those of normal arteries (345). Similarly, oxidative DNA damage and DDR markers appear in atherosclerotic lesions in animal models after fat feeding and in human plaques, and whilst some markers are reduced by lipid lowering, oxidative DNA damage persists (346;347). Finally, it is common to

observe DNA damage not only in genomic but also in mitochondrial DNA in atherosclerosis. For example, circulating cells in patients with severe coronary atherosclerotic disease exhibit a significantly higher incidence of the common mitochondrial deletion MtDNA 4977 (348), normally observed in aged cells.

Over and above the role of the DDR, telomere length biology is another aging pathway which seems to play a central role in initiation and evolution of atherosclerosis. Shortened telomeres are evident in atherosclerosis, observed in plaque vascular smooth muscle cells (349) and endothelial cells (350) relative to the normal vessel wall, as well as in circulating endothelial progenitor cells (351). Also, LTL is shorter in patients with atherosclerosis compared with control subjects (61;352) and it is inversely correlated to CV mortality and disease risks (61;353;354).

2 AIMS

The biology of LTL and its relationship with chronic inflammatory and oxidative stress exposure remains largely unclear in humans. Several epidemiological studies reported conflicting results on these associations. The purpose of this thesis was to explore systematically the association between LTL, clinical measures of chronic inflammation and oxidative stress exposure in humans. Furthermore, the relevance of these pathways in the evolution of age-related disease was examined.

The specific aims of this project were to:

1. Explore the association between markers of chronic inflammation and oxidative stress exposure with LTL.
2. Investigate the relationship between measures of chronic inflammatory burden and LTL and their dependence on the metabolic status at the time of the evaluation.
3. Define whether the adult association between shorter LTL and inflammatory markers is detectable at younger ages, further supporting the hypothesis that long term inflammatory burden is the main driver of LTL shortening throughout post-natal life.
4. Define the contribution of the post-natal regulation of the rate of LTL shortening in the evolution of age-related disease (i.e. atherosclerosis).

3 GENERAL METHODS

3.1 Vascular Measures

The major problem in exploring the mechanisms implicated in CVD evolution however, relates to the long preclinical phase of the disease, so that the role of aging pathways on atherosclerosis is difficult to evaluate. Over the last twenty years, several non-invasive techniques have been developed to monitor changes of the vascular wall induced by atherosclerosis, enabling study of the pathways involved in disease initiation and evolution. Increased intima-media thickness and reduced vascular elasticity are common features of both vascular aging and atherosclerosis and can be easily measured using ultrasound techniques.

3.1.1 Common carotid artery intima-media thickness

Over the past decade the measurement of carotid artery intima-media thickness (cIMT) using high resolution B mode ultrasonography has emerged as a useful measure of atherosclerosis and its progression and for assessing CV risk. Measurement of cIMT is non-invasive, safe and well accepted by patients and it has been associated with the presence and extent of coronary atherosclerosis (355). In 1986, Pignoli et al (356) demonstrated that measures of cIMT by ultrasound were closely correlated with histology (357), although some studies have documented that ultrasound estimation gives a slighter higher cIMT reading (356). The confirmation that cIMT is on the causal pathway for atherosclerosis is provided by several lines of evidence.

1. Increased cIMT has been associated with higher risk of CVD. The Kuopio Ischaemic Heart Disease Risk Factor (KIHD) study (358), conducted in 1,257

eastern Finnish men, with a follow-up period of 2 years, evaluated ultrasonographic carotid findings with the risk for acute myocardial infarction. The presence of plaque was associated with an increased relative risk of 4.1 (95% confidence interval [CI] 1.8 to 9.2) compared to individuals free from plaques. There was a 2.1-fold increased risk for myocardial infarction with increased cIMT >1 mm, compared to men free of these atherosclerotic lesions, and if used as a continuous variable, there was an 11% increase in risk for myocardial infarction with each 0.1-mm increase in cIMT. Differences between men and women in the association of cIMT and coronary artery disease (CAD) incidence were demonstrated in the Atherosclerosis Risk in Communities (ARIC) study (359). This study was conducted in middle-aged men and women free of disease at baseline, with a follow-up period of 4 to 7 years. Hazard ratios for cIMT >1 mm adjusted for CV risk factors were 2.62 (95% CI 1.55 to 4.46) for women and 1.20 (95% CI 0.81 to 1.77) for men. The Rotterdam study (360), however, found that the risk of myocardial infarction in women and men was similar, with odds ratios adjusted for CV risk factors of 1.26 (95% CI 0.89 to 1.79) and 1.25 (95% CI 0.91 to 1.72), respectively. More recently, investigators of the Carotid Intima Media Thickness [IMT] and IMT-Progression as Predictors of Vascular Events in a High Risk European Population (IMPROVE) Study showed that not only cIMT but also morphological changes of the common carotid artery (e.g. increased intra-adventitia diameter) are strong predictors of CV events, independent of traditional CV risk factors included in the Framingham risk score (361).

2. Exposure to well established CV risk factors has been associated with increased cIMT and that cIMT values have also been shown to be responsive

to pharmaceutical interventions. Several studies have shown that diabetes mellitus is associated with an increased cIMT and that duration of type 2 diabetes is also an important determinant of increased cIMT (362;363). Increased cIMT has also been reported in individuals with hypercholesterolemia (364). Of all the traditional CV risk factors, hypertension has the greatest impact on cIMT, and this is due presumably to the associated medial hypertrophy (365;366). The association of cIMT with CV risk factors is evident from young age, as it has been recently demonstrated in the Bogalusa Heart Study (367). Therapeutic interventions including treatment with blood pressure lowering agents, lipid lowering agents as well as multifactorial interventions in high risk patients can slow the progression or even reduce cIMT (368-371). The Pravastatin, Lipids, and Atherosclerosis in the Carotid Arteries II (PLAC-II) study (372) demonstrated a lesser progression of cIMT with pravastatin compared to placebo (0.0295 and 0.0456 mm/year, respectively). Similarly, a reduction in the progression of cIMT was reported following treatment with lovastatin, with a reduction of mean maximum cIMT of −0.009 mm/year compared to the progression of cIMT in the placebo group of 0.006 mm/year (373). The Measuring Effects on Intima-Media Thickness: An Evaluation of Rosuvastatin (METEOR) trial reported similar results, demonstrating that rosuvastatin therapy resulted in a reduction of the progression of maximum cIMT compared to placebo in middle-aged adults with low Framingham risk scores (FRS) and subclinical atherosclerosis, with changes of −0.0014 and 0.0131 mm/year, respectively (374). Furthermore, while the Monitored Atherosclerosis Regression Study found that dietary cholesterol, body mass index and smoking were important

determinants of the annual progression of cIMT, lifestyle modifications such as weight loss and smoking cessation have been associated with 0.13mm/year reduction in progression of cIMT (375).

cIMT measurements are not only predictive of atherosclerosis evolution but can also inform on the physiological modifications of the common carotid artery. For instance, cIMT can be measured serially and this approach has demonstrated that cIMT increases significantly with age. Rates of progression in control groups have ranged from 0.006mm/year in asymptomatic to 0.06mm/year in individuals with CAD (376;377). Post-mortem studies indicate that the aortic wall thickening that occurs with aging consists mainly of intimal thickening, even in populations with a low incidence of atherosclerosis (336). Non-invasive measurements made within the context of several epidemiological studies indicate that cIMT increases 2- to 3-fold between 20 and 90 years of age, which also is the case in individuals rigorously screened to exclude carotid or coronary arterial stenosis (378). There is, however, a marked heterogeneity in cIMT among individuals of a given age. Although arterial remodelling with aging in otherwise healthy humans occurs in the context of age-associated endothelial dysfunction (379), there is presently little information on the factors involved in progressive IM thickening with aging in humans. Gender and ethnicity are considered important non-modifiable factors influencing differences in cIMT. Several studies have reported thicker cIMT in men compared to women (379;380) and the Insulin Resistance Atherosclerosis Study found that ethnicity may also influence cIMT, which is greater in blacks, lower in Hispanics, and intermediate in whites (381-383).

3.1.1.1 Experimental technique

Different ultrasound techniques have been used to measure cIMT. Although M-mode (Figure 3.6) has superior temporal resolution, this provides measurements of only a single point of thickness, rather than a segmental value.

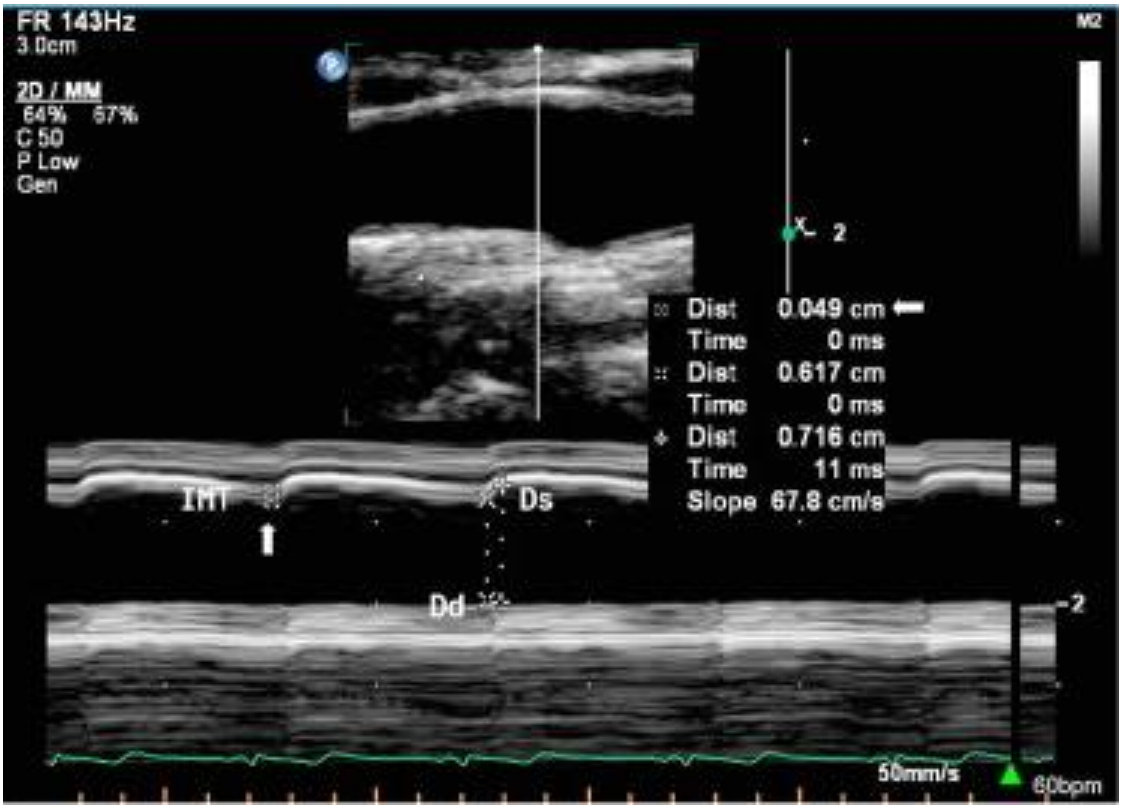


Figure 3.1 Example of M-mode cIMT acquisition.
Although M-mode images provide superior temporal resolution compared to B-mode, the main limitation of this method of acquisition relates to the single point used to estimate cIMT. This considerably increases the chance of error during acquisition and reduces measurement precision.

As carotid wall thickening is not uniform, a single value may not accurately represent the real cIMT value. Furthermore, M-mode or point-to-point measurements of B-mode images provide estimates of cIMT which are limited multiples of the pixel size. This increases the chance of errors and reduces accuracy in defining cIMT values, as changes in the structure of the arterial walls can have sub-pixel magnitude. In contrast, multiple measurements of several extended segment lengths reduce the chance of error and allow expression of cIMT values with higher precision (sub-

pixelar level). Recently, echotracking devices equipped with semi-automated border detection programs have been used to measure cIMT (see below), and can calculate the average IMT during whole the cardiac cycle or separately for the systolic and diastolic phases (384). Measurements, however, are best acquired at the end-diastolic phase, because the systolic expansion of the lumen causes cIMT thinning (385).

A number of different scanning procedures (reference site, arterial wall, direction of scanning) have been used to determine IMT. Most of the studies have measured IMT in the carotid artery but the best site of intima-media thickness (IMT) measurement is still a matter of debate. The common carotid artery (CCA) (1-2 cm proximal to the carotid bulb) has been examined in most studies whereas the internal carotid artery (ICA) and carotid bulb have been seldom studied (386). The CCA is easier to image as it is relatively close and parallel to the skin surface. In contrast IMT measurements in the ICA are more challenging (387). Although atherosclerotic lesions appear later in the CCA compared to ICA or bifurcation, the IMPROVE study demonstrated that changes in all sites seem to be equally strongly associated with risk of subsequent CV events (361). Apart from the carotid artery, other arterial sites such as the common femoral, brachial or radial arteries have been proposed for IMT measurements. While in the elastic CCA IMT is assumed to represent mainly intimal thickening, IMT of the more muscular peripheral arteries represents both a change in the intima and /or media layer (384). There is conflicting evidence on the predictive role of IMT thickening in the muscular arteries for future risk of CVD. The Angina Prognosis Study in Stockholm (APSIS) showed that IMT of the common femoral artery and plaques were related to the risk of CV death or myocardial infarction, while femoral IMT was related to CV death or myocardial infarction, as well as to revascularization. However, after adjustment for age, sex, smoking, previous CVD

and lipid status, cIMT failed to predict any CV event, whereas carotid plaques tended to predict the risk of CV death or myocardial infarction. Femoral IMT and plaques were also related to the risk of revascularization after adjustments (388). Other groups have reported an association between ventricular hypertrophy and IMT of both the common carotid and the brachial artery in patients with previous myocardial infarction. Another study showed that increased blood pressure was associated with increased IMT of the femoral artery but not the carotid artery (389). As a result, the CCA remains the preferred site for IMT measurements (384). A recent consensus Statement from the American Society of Echocardiography recommended that ultrasound images of the distal 1 cm of the far wall of each CCA should be obtained and compared with values from a normative data set.

There have been a variety of cIMT protocols published with differences in scanning directions of CCA, corresponding to different views of the vascular wall (anterolateral/lateral/posterolateral). Acquisition of multiple views provides information on wall-thickness eccentricity (390). Most studies have measured cIMT of the far wall, while others averaged these measurements with those of the near wall (391). Importantly, one study, evaluated the accuracy of the ultrasound measurement of the near wall cIMT and found that this was 20% lower than the corresponding histologic measurement (392). Therefore, far wall images are more easily obtained, accurate and standardized.

3.1.1.2 Experimental protocol

The protocol used to measure cIMT in this thesis follows recent guidelines on cIMT measurement (380). Images were obtained with the subject laying supine in a temperature controlled room with slight hyperextension and rotation of the neck to the

contralateral side. A sonographer was positioned on top of the bed, behind the head of the patients (Figure 3.7A).

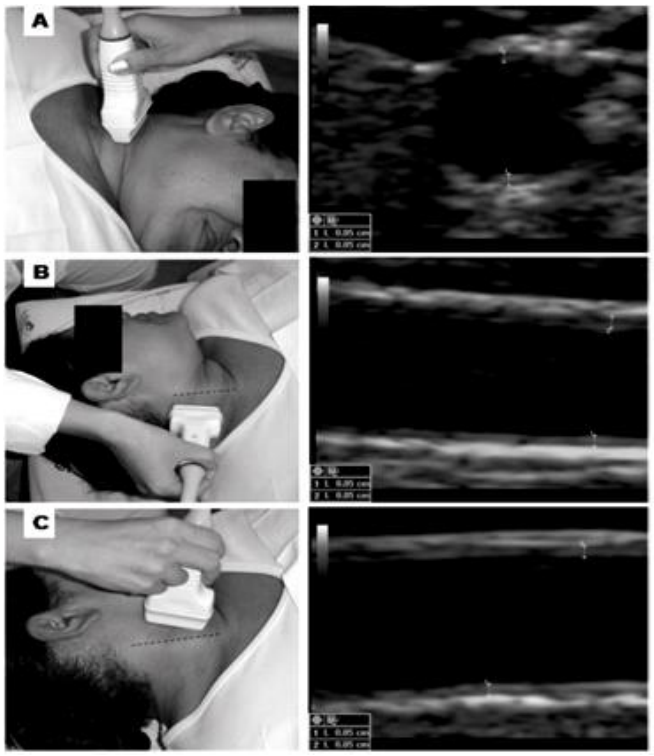


Figure 3.2 cIMT acquisition technique.
A) The subject lay supine with slight hyperextension and rotation of the neck to the contralateral side of acquisition. The sonographer (positioned on top of the bed, behind the head of the patients) places a high resolution ultrasound probe on the middle of the patient's neck. The proximal part of the carotid bulb was identified in transversal plane. This allowed identification of the region of interest of the common carotid artery, 1cm below the bulb. B) With slow movements of the probe, the longitudinal axis of the artery was visualised. C) Images were optimised to obtain the best view of the intima-media thickness of the far arterial wall. The optimal longitudinal image was acquired continuously for 10 seconds and video-clips were stored for post-acquisition analysis.

Images of both the right and left carotid arteries were obtained with linear-array transducers with frequencies of 12 MHz, connected to a high-resolution ultrasound scanner (Vivid I or Vivid 7, GE Healthcare). The proximal part of the carotid bulb was identified, initially with a transversal plane to identify the segment of the common carotid artery 1 cm proximal to the bulb. The transducer was manipulated so that the near wall of the carotid artery was parallel to the transducer

footprint and the lumen maximised in the longitudinal plane (Figure 3.7B). The image was focused on the far wall and the zoom function was used to magnify the region of interest. The optimal longitudinal image was acquired continuously and recorded in DICOM format for 10 seconds to minimize variations. Using different angles of the ultrasound probe, images of the left and right common carotid artery were acquired with three different views: anterior, posterior and lateral (Figure 3.7 B and C). As the cIMT measurements were captured on the far wall, the use of different views during the acquisition allowed the measure of the posterior, anterior and medial cIMT, respectively. Once acquired, all recordings were analysed offline using an echotracking software equipped with semi-automated border detection system (Carotid Analyser, Medical Imaging Applications, Iowa, USA).

3.1.1.3 Analysis of cIMT cineloops

Cineloops were opened using the Carotid Analyser software and calibration was applied to define which distance on the image corresponded to the size of 1 cm. Following calibration, a region of 5 cm located 1 cm blow the carotid bulb and including both vascular walls was selected for analysis (Figure 3.8).

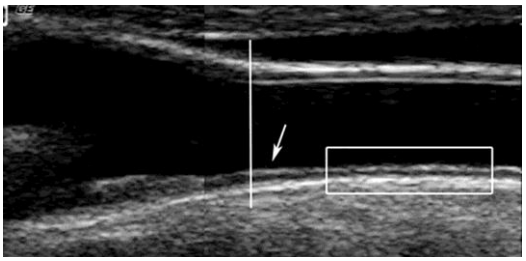


Figure 3.3 Region of interest for cIMT measurements.
A segment of approximately 5cm of CCA, located 1cm below the bulb was used for estimation of cIMT.

An automatic edge detection line was placed at the interface between media and adventitia of both the proximal and distal vascular walls (Figure 3.9, purple lines). Once the cineloop was started, the software placed a second automatic edge detection line at the interface between intima and vascular lumen (Figure 3.9, yellow lines).

This allowed the software to automatically calculate, frame by frame: a) the intima-media thickness (space included between the line defining the intima-vascular lumen interface and that defining the medial-adventitia interface) and b) the intra-adventitia lumen (space included between the lines defining the medial-adventitia interface on both vascular walls) (Figure 3.9).

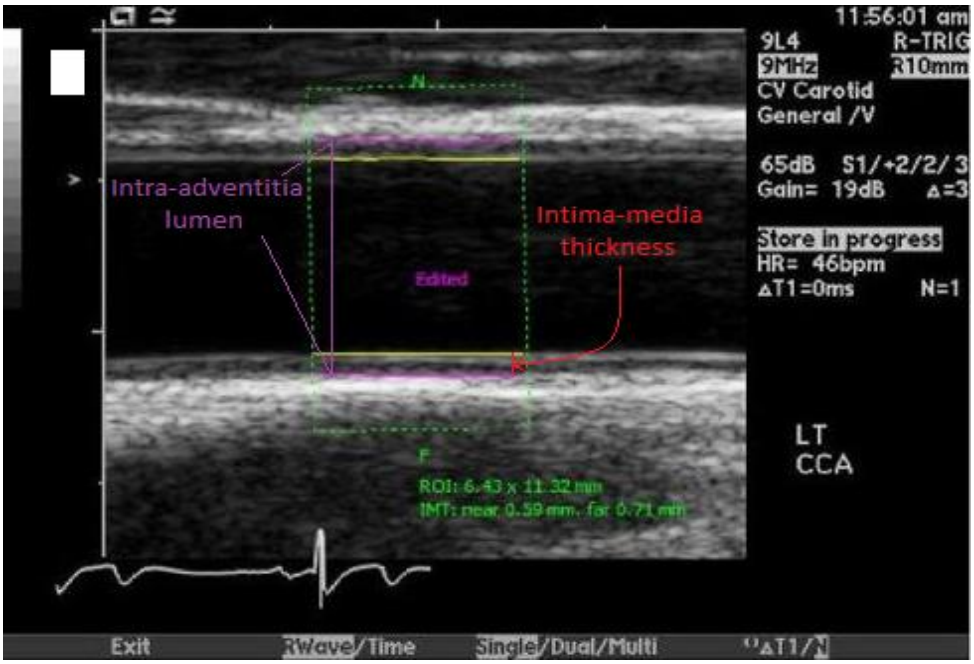


Figure 3.4 Automatic edge detector.
Once the region of interest was identified an automatic edge detection line was placed between the media and adventitia (purple). Once the cineloop was started, a second detection line (yellow) was placed by the software at the interface intima-lumen. The distance between the purple and yellow lines defined the intima media thickness during analyses.

As the software processes the cineloops frame by frame, at the end of each reading the information on the IMT and intra-adventitia lumen are provided as an average of the 10 seconds of recording as well as at end systole and end diastole. The end-diastolic frame of three consecutive cardiac cycles with a clear definition of the intima-media thickness were averaged to provide the final values of cIMT for the three different acquisition views.

3.1.1.4 Reproducibility

Phantom studies have shown that distances similar to the cIMT can be measured with B-mode ultrasound system with an axial resolution of 0.2 to 0.4mm at a precision of about 0.03 to 0.05mm (393). Reported intra-observer variability of cIMT has been reported between 2.4% to 10.6 % and inter-observer variability varies from 3.1% to 18.3% (394). Reproducibility of IMT measurement is better in studies limited to the common carotid artery far wall than in those which have included multiple measurements at different carotid sites. In addition, variability is less when mean IMT is measured compared to maximum IMT and also when measurements have been performed in more than one direction (395). Recently, the studies with an automated computerized IMT measurement rather than a manual cursor placement have reported the best reproducibility (395).

3.1.2 Brachial artery distensibility

Vascular remodelling results in changes in arterial elasticity which reduces the ability of the vessel to respond to changes in local blood pressure. Vascular elasticity is assessed by arterial compliance and distensibility.

Compliance is defined as the change in volume per unit of pressure ($\Delta V/\Delta P$) and reflects the buffering function of the vessel (396;397). Arterial compliance is an important determinant of the afterload on the heart (396); a decrease in total arterial compliance contributes to a higher afterload. Arterial compliance (C) is related to arterial distensibility (D) and arterial volume (V) by the formula $C=D\times V$ (397). Distensibility, defined as the relative change in volume per unit of pressure ($[\Delta V/V]/\Delta P$), is related to the elastic Peterson modulus (398) and is a determinant of stress on the vessel wall.

Distensibility of large and middle-size arteries is an important measure of CV phenotype (399), and a reduction in arterial distensibility causes: 1) an increase in arterial impedance, cardiac work and cardiac oxygen consumption, 2) a reduction in the diastolic portion of vital organ perfusion which is particularly pronounced for the coronary circulation and 3) an increase in systolic blood pressure, pulse pressure and indirectly or directly-generated trauma on the vessel wall with a resulting progression of atherosclerosis (397;398).

Measuring arterial distensibility has always been difficult because of the need to assess accurately and concomitantly changes in vascular volume in response to changes in pressure over a wide range of values. This has led to a variety of indirect approaches, such as measurements of the 1) pulse wave velocity which varies in proportion with the stiffness of the vessel wall throughout the arterial tree 2) blood pressure and blood flow contour in a peripheral artery or the aorta to derive, particularly from the diastolic phase, the elastic recoil of the vessel and 3) diameter/pressure relationship in accessible arteries, to have measurements of local distensibility from which to infer more generalized changes in different conditions and diseases. The last approach has gained popularity because of the possibility to track accurately, noninvasively and continuously changes in arterial diameter in response to blood pressure changes in a number of vessels.

Pulsed Doppler systems can estimate, transcutaneously, the time-dependent changes in arterial diameter relative to its initial diameter by assessing the displacement of the arterial walls during the cardiac cycle (distension waveform) (400-402). The displacement of the arterial wall can be recorded on a two-dimensional B-mode echo-image by processing radiofrequency signals within a sample volume (either the vessel walls itself or the wall-blood interface) identified

along an M-line perpendicular to the artery of interest. The distension waveform of the artery normalized with respect to the local pulse pressure provides information about the local elasticity of the arterial wall (Figure 3.10).

Figure 3.5 Ultrasound evaluation of resting arterial distensibility with radiofrequency (RF) echo tracking. *Adapted from Urbina et al. Hypertension 2009;54:919-950.*

Using this system, it has been established that the distensibility and compliance of the common carotid artery gradually decrease with age (403;404) as well as that arterial distensibility is impaired in adult patients with CV risk factors, including hypercholesterolemia (405), arterial hypertension (406-408), and diabetes mellitus (409). The high sensitivity of radiofrequency systems to analyse B-mode echo-images has also been used to show that brachial artery distensibility is reduced in children with higher levels of LDL cholesterol as well as in adolescents with adiposity and metabolic syndrome (410;411).

Importantly, evidence is available that reducing the CV risk factor burden can improve arterial distensibility. The hypertension related changes in large artery distensibility can be improved by long-term antihypertensive treatment both in

isolated systolic and essential hypertension (412). It has been suggested that the increase in compliance with calcium antagonists (413) and ACE inhibitors (414) is mainly caused by an increase in distensibility, with a minor influence on large artery diameter. In contrast, isosorbide dinitrate increases the compliance of the carotid and brachial arteries mainly through an increase in large artery diameter (415). The effect of antihypertensive agents may also depend on the vascular territory. The diuretic amiloride/hydrochlorothiazide did not change carotid artery wall properties but did increase brachial artery wall properties (414). Long-term lipid-lowering treatment can improve the striking arterial stiffening in familial hypercholesterolemic individuals (405).

The high sensitivity of radiofrequency systems to analyse B-mode echo-images, the non-invasive and relatively easy acquisition protocols as well as the ability of arterial distensibility to reflect the level of CV risk factor burden in large epidemiological studies, have made this approach an attractive test to follow vascular remodelling, particularly in childhood and adolescence (as in study 3).

3.1.2.1 Acquisition protocols and reproducibility

Measurements were performed at rest, in a temperature controlled room. The subject lay supine on a couch, and after 10 minutes' rest, the right brachial artery was imaged in longitudinal section 10 to 15 cm above the antecubital fossa with a 7-MHz linear array transducer and Acuson 128XP/10 (416). To measure arterial distension, the M-mode cursor was positioned at right angles to the arterial lumen over the clearest defined section of the artery on the B-mode image. A 5-second segment of the radiofrequency signal was recorded by a separate commercially available Wall Tracking System (Ingenious Medical Systems) at a rate of 800 Hz (1 frame/ms). The

initial frame (amplitude waveform) was displayed, and the operator selected two sample volumes coinciding with the arterial wall-lumen interfaces. The relative position of the walls within these volumes was measured every 25 ms (≈20% of the expected time for an upstroke) by use of a displacement detection algorithm based on the cross-correlation model for corresponding segments of the radiofrequency lines (Figure 3.11) (400).

Figure 3.6 Example of radiofrequency signal to estimate brachial artery distensibility
Example of wall tracking output, showing movement of anterior and posterior walls and the relative changes of arterial diameter (estimated as distance between the two vascular wall, below) during 4 cardiac cycles. Adapted from Whincup at al. Circulation 2005.

Arterial distension is the mean diameter change between diastole and systole over the 5-second period. (417). Pulse pressure was measured in the left brachial artery with a Dinamap 1846SX oscillometric blood pressure recorder (Critikon Inc) at the same time that distensibility was measured in the right arm. This method has been shown to provide a representative measure of the pulse pressure in the right brachial artery during data collection (400). Coefficients of variation for diameter and distension measurements are 2% - 3% (417;418).

3.2 Oxidative Stress Assays

Most available methods for evaluating oxidative stress *in vivo* are inappropriate for large-scale use, limiting their application in clinical practise (266;419;420). The choice to use one specific biomarker as representative of the total oxidative stress or anti-oxidant state in specific patient categories is still controversial (266;419;420). Indeed, each disease can be characterized by increased production of different oxidative species, depending on their intracellular sources. While electron spin resonance (ESR) is currently considered the gold standard assay to measure different RS in biological samples (264), the method is complex and not available in most clinical laboratories. Furthermore, ESR uses different protocols depending on which oxidative species are targeted, requiring continuous adaptation of the experiment and machine parameters and introducing further complexity in the measurements. Ideally, the perfect oxidative stress assay should be easy to perform, reliable, quick and inexpensive. Automated analysers would allow processing of a large number of samples, avoiding manual sample and reagent handling, and reducing variability sources.

Among the various commercially available kits, the reactive oxygen metabolites (dROM) and biological antioxidant potential (BAP) tests have been developed to assess in an automated, economic and reproducible fashion the total amount of oxidant and antioxidant species in different biological samples.

3.2.1 d-ROM Test

The d-ROMs test measures the blood concentration of hydroperoxides, a class of chemical oxidant species belonging to the wider group of reactive oxygen metabolites (421-423).

Hydroperoxides are generated by the oxidation of several molecules such as glucosides, lipids, amino acids, peptides, proteins, and nucleotides, making their levels independent of the source of oxidative stress. In the presence of free iron, hydroperoxides are able to generate alkoxyl and peroxyl radicals, according to the Fenton's reaction (424;425):

$$ROOH + Fe^{2+} \rightarrow RO\bullet + OH^{-} + Fe^{3+}$$

$$ROOH + Fe^{3+} \rightarrow ROO\bullet + H^{+} + Fe^{2+}$$

Such radicals are highly reactive and can quickly oxidise surrounding positively charged molecules, in order to recover their electron stability. By adding to the solution a stable and positively charged radical (N,N-diethylparaphenilendiamide radical) which rapidly interacts with the alkoxyl and peroxyl radicals becoming pink after oxidation, the d-ROM test photometrically measures the amount of alkoxyl and peroxyl radicals formed by degradation of hydroperoxides (423).

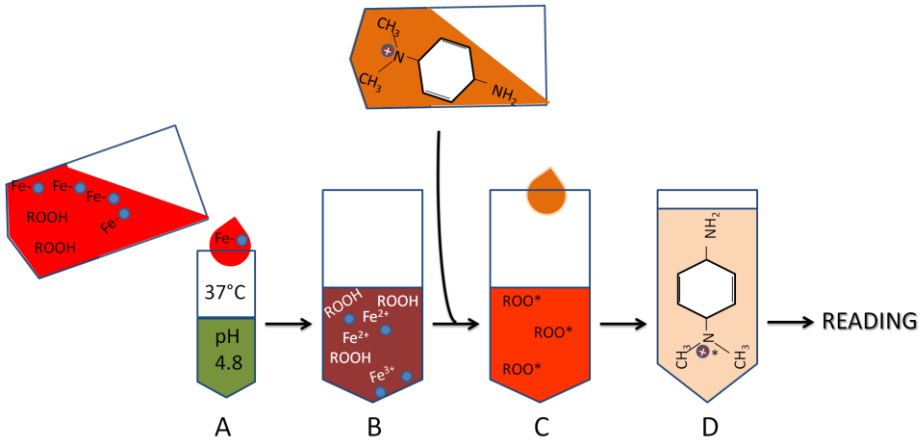


Figure 3.7 d-ROM test.
The reaction is started by adding a small amount of serum to a solution containing N,N-diethylparaphenilendiamide radical and an acidic buffer (pH 4.8). The solution is warmed up to 37°C. This detaches the iron ions from serum protein, making them available for the Fenton reaction and transforming the hydorperoxides in the samples in alkoxyl and peroxyl radicals. The latter rapidly react with the N,N-diethylparaphenilendiamide radical, causing a change in the colour of the solution which shifts to pink. The intensity of the pink colour is directly related to the original amount of hydroperoxides in the sample and is readable using a photometer with absorbance at 505 nm.

Assay: 20μL of serum are diluted in a solution formed by: a) 10μL of a chromogenic mixture containing 10% of the positively charged N,N-diethylparaphenilendiamide radical and b) 1ml of a buffer containing HCl with pH 4.8 (Figure 3.12 A and B). The sample is warmed at 37°C for 1 minute. The combination of the high temperature and acidic environment allows detachment of iron ions from serum proteins, making them available for the Fenton reaction (Figure 3.12 B and C). Thus, the hydroperoxides in the samples are transformed in alkoxyl and peroxyl radicals which rapidly react and oxidize the N,N-diethylparaphenilendiamide (Figure 3.12 C and D). This determines a progressive change in the colour of the solution (towards pink) which can be read by a photometer (absorbance at 505 nm) (Figure 3.12 D). The final reading is dynamic and is performed immediately as well as at 1, 2, and 3 min following incubation. Obviously, the level of absorbance is directly dependent on the amount of the reactive oxygen metabolites present in the serum, according to the Lambert–Beer's law (423). A blank reagent obtained by replacing serum with distilled water and a standard with assigned value are included for each series of assays. The results of d-ROM test are expressed in arbitrary units called "Carratelli Units" (CARR U), according to the following formula:

$$\text{CARR U} = F(\Delta\text{Abs/min})$$

where *F* is a correction factor (approximately 9000 at 37°C according to the results obtained with the standard); (ΔAbs/min) are the mean differences of the absorbances recorded at 1, 2, and 3 min. Reference values of healthy subjects are between 250 and 300 CARR U; conditions of slight, medium, and high oxidative stress are defined,

respectively, by values of 320–360, 360–400, and >400 CARR U; for values up to 500 CARR U, a sample dilution is required (426). Results obtained with d-ROM test have been validated by electronic spin resonance spectroscopy and it has been experimentally established that 1 CARR U corresponds to 0.08 mg of H_2O_2/dl (423;426). Furthermore, it has been proved that, in vertebrate blood samples, results are stable also after refrigeration of the sample (427).

In this study, the intra-assay coefficient of variation obtained by running 20 randomly selected samples in duplicate was 3%. All analyses were performed in a blind fashion.

3.2.2 BAP test

This method evaluates the reducing power of a biological sample. A salt of trivalent iron FeCl3 is dissolved in a colourless solution containing thiocyanate which turns its colour to red due to the action of ferric ions. This solution is oxidatively saturated (all ferric ions are trivalent) and can be decolorized by the addition of blood serum due to the reduction of ferric ions to bivalent ions (Fe^{2+}) caused by the action of serum antioxidants. The antioxidant potential of serum can be then evaluated by assessing photometrically the degree of decolorization which reflects the amount of reduced ferric ions i.e. the reducing capacity of blood (428-434). A sample of lyophilized serum with a known antioxidant capacity (provided with the kit) and 10 µL of distilled water are used for calibration of the results.

Assay: The procedure starts solving the lyophilized serum in 2mL of distilled water. It is then necessary to prepare three tubes mixing all reagents according with the following table:

Table 3.1 Reagents used for BAP test

	Blank solution	Sample	Calibrator
Thiocyanate solution (Reagent 1)	1ml	1ml	1ml
FeCl3 salt (Reagent 2)	50 µL	50 µL	50 µL
Distilled water	10 µL	-	-
Serum of interest	-	10 µL	-
Solved lyophilized serum	-	-	10 µL

After 5 minutes of incubation at 37°C, the absorbance at 550nm of the distilled water sample will be established by photometric reading and used as 0. Then, the decolorization of the other two samples will be measured and the intensity of this change will be directly proportional to the ability of the two serum samples (calibrator and tested sample) to reduce ferric ions (435).

The results are obtained according to the following formula:

$$\frac{\text{Absorbance of blank sample} \cdot \text{Absorbance of tested sample}}{\text{Absorbance of blank sample} \cdot \text{Absorbance of calibrator}} \bullet \text{Calibrator}$$

and expressed in units of µmol/L (µmol of ferric ion reduced per L of sample). Normal reference values of BAP test are >2200 µmol/L. Values below 2000 µmol/L indicate an antioxidant deficiency status. The advantage of BAP test is that it provides a global measurement of many antioxidants, including uric acid, ascorbic acid, proteins, α-tocopherol and bilirubin (435). As it is easy to perform, has a good reproducibility, is cheap and can be easily applied to large population studies, the BAP test was used in the first study of this thesis to assess the biological antioxidant

potential of the blood of subjects with and without periodontitis. All analyses were performed in a blind fashion. In the interpretation of the results, it should be recognised, however, that the BAP test has major limitations:

- Whilst most antioxidants are able to reduce ferric ions to ferrous ions, not all are able to do so. This means that some antioxidants (such as carotenoids and SH-group antioxidants) will not be determined by this assay.
- Substances other than antioxidants, are able to reduce the ferric ions into ferrous ions. These will cause falsely high results being obtained as these are not antioxidants (e.g. glucose, hydroperoxides, ethanol).Since the effects of proteins are weak, the assay practically measures non-protein total antioxidant capacity

3.3 LTL assay

3.3.1 Sample preparation

3.3.1.1 DNA extraction

The DNA extraction in this thesis was performed using a modified "salting-out" method reported by Miller et al (436), unless otherwise specified. This method is based on 5 different steps: cell lysis, nuclear lysis, deproteinisation, DNA extraction, DNA precipitation and DNA recovery. Twenty millilitres of ice-cold cell lysis buffer (consisting of 0.32M sucrose, 5mM MgClg, 10mM Tris-HCI with pH 7.5, 1% Triton-X-100) were added to a 30 ml polypropylene tube containing 3-5 ml of whole blood. After inversion for 10 times to ensure thorough mixing, the tube was centrifuged at

1300g at 4°C for 10 minutes and the supernatant was discarded without disturbing the pellet. This washing step was repeated by re-suspending the pellet in 20ml of cell lysis buffer. Following the second washing step the pellet were re-suspended with a Pasteur pipette in 2ml of a nuclear lysis solution, containing 10 mM Tris-HCI (pH 8.2), 0.4 M NaCi, 2mM Na_2EDTA (pH 8.0). After inversion for several times, 1 ml of a deproteinisation solution consisting of 5 M sodium perchlorate was added to the sample. The tube was placed on a shaker and the sample left in incubation for 30-45 minutes. After incubation, 2ml of ice-cold chloroform (-20°C) was added to the sample using a glass pipette and the tube was inverted several times and centrifuged at 1300g for 3 minutes at room temperature. The upper aqueous phase was recovered without disturbing the organic phase and transferred to a fresh 30ml polypropylene tube, using a Pasteur pipette. Ten millilitres of cold (-20°C) 100% ethanol was slowly added to the sample and, after a few minutes, the tube was inverted several times to precipitate the DNA. The white sediment of DNA was recovered with a sterile Pasteur pipette and quickly washed in 70% ethanol. The tip of the Pasteur pipette was placed into a 1.5 ml apex sterile tube containing 1 ml of Tris-EDTA (TE) buffer (10mM Tris, 1mM EDTA) and cut proximal to the top of the tube. This was sealed and left incubating overnight at 37°C to allow complete dissolution of the DNA. Then, the samples were kept at 4°C for a minimum of 2 weeks to allow the viscous DNA to dissolve.

3.3.1.2 DNA Quantification

In all studies, the DNA concentration was measured with a Nano-Drop spectrophotometer, according to a standardised protocol (437). Absorption

spectroscopy has long been the method of choice to measure the amount of DNA or RNA in a solution (438). Purines and pyrimidines in nucleic acids absorb ultraviolet (UV) light because of presence of conjugated double bonds of the constituent purines and pyrimidine bases, which have maximal absorbance at wavelengths of 260 nm. Therefore, if a light source shines on a sample, the amount of light that passes the sample can be measured, and the amount of light absorbed by the sample can be inferred (439-441). For double stranded DNA, one optical density of 1 unit (normally indicated as OD260 or A260) corresponds to concentration of 50μg/ml, while for single stranded DNA and RNA it is equivalent to 40 μg/ml. Using the Lambert-Beers law which relates the absorption of light to the properties of the material through which the light is travelling, it is therefore possible to calculate by simple interpolation the concentration of nucleic acid in a specific solution. Furthermore, using spectrometry is possible to check the presence of contamination by organic compounds or proteins. Based on the same principle of the DNA, proteins normally absorb light at wavelengths of 280 nm while organic compounds absorb at 230 nm. Therefore, the A260/280 and A260/230 ratios define the purity of each DNA sample extracted compared to proteins and organic compounds, respectively (439-441).

The Nanodrop spectrophotometer is composed of a sampling arm with two pedestals (top and bottom, Figure 3.13 A).

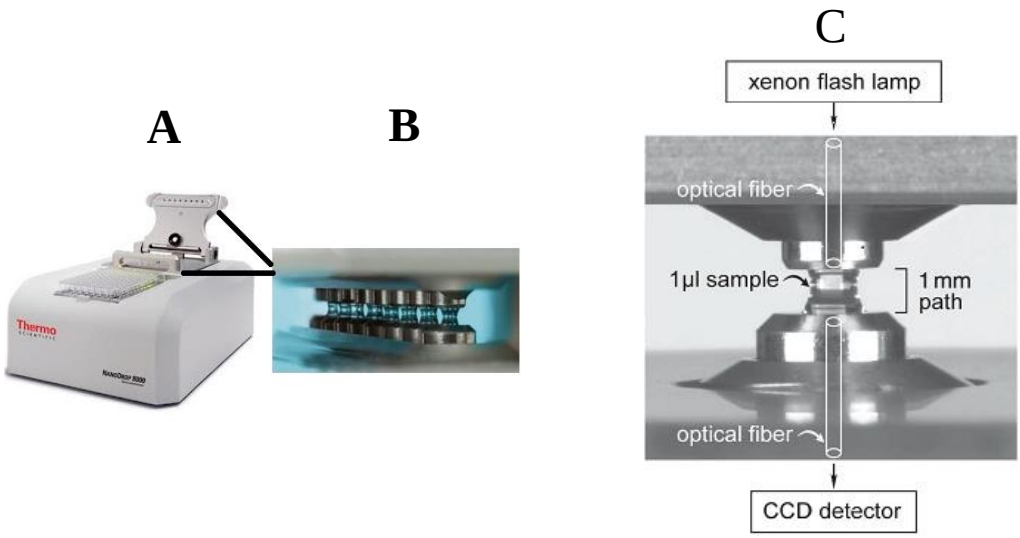


Figure 3.8 NanoDrop structure.
A) With the sampling arm open, the sample is pipetted on to the lower measurement pedestal. B) The sampling arm is closed and the sample remains automatically drawn between the upper and lower measurement pedestals. Measurement initiates using the operating software on the PC. C) UV light is emitted by a xenon flash lamb located on the top pedestal and passes through the sample. A charge coupled device (CCD) detector records the residual amount of light on the bottom pedestal.

On the top pedestal a xenon flash lamp emits light which is transferred through the sample by optical fibres (Figure 3.13 B). Once passed the sample, the unabsorbed light is captured by a charge coupled device (CCD) detector located in the bottom pedestal, which transforms incident photons in an electric charge readable and quantifiable by electronic systems (Figure 3.13 C) The difference between emitted and captured light informs on the amount of absorbance at different wavelengths therefore defining the concentration of different compounds in the interrogated solution.

Assay: Samples were removed from the freezer and thawed in the refrigerator overnight to avoid excessive evaporation of the TE/DNA solution. On the morning of the following day, cryovials were centrifuged for 1 minute at 3000rpm and transferred in 96 well plates under sterile conditions. These plates constituted the stock DNA solutions. In each well, DNA was homogeneously re-suspended in the TE solution by

gently pipetting up and down with a multichannel pipette. Using other sterile 96 well plates, a working solution was prepared for each sample by diluting 10 µl of the stock solution with 100 µl of nuclease and protease free water (Sigma). This further dilution reduced the amount of DNA lost for quantification, also provides a more accurate reading for those samples with excessively high concentration of DNA. Stock plates were re-stored in a -80°C freezer, while working solutions were left to stand for 15-20 minutes at room temperature to ensure the complete diffusion of DNA throughout the water. While waiting, the computer and Nanodrop spectophotometer were turned on to allow the xenon lamp to warm up for approximately 15 minutes before reading samples. With the sampling arm opened, the upper and lower pedestals were cleaned using a soft laboratory wipe (Kimwipe) soaked with distilled water.

Any measurement session was begun with a blanking cycle (using distilled water) to assure that the instrument was working properly and that the pedestal was clean. When the reading evidenced the presence of contamination, further cleaning and blanking cycles were performed until the spectrum was completely flat. Between each measurement, both pedestals were cleaned using soft laboratory wipes (Kimwipe, Kimberly Clark, USA). Once the blanking procedure was completed, 1.5 µl of each sample (working solution) was sequentially loaded onto the lower measurement pedestal (Figure 3.13 A). The sampling arm was closed and a spectral measurement was initiated using the operating software on the PC (Figure 3.13 B). The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement made (Figure 3.13 C). When the measurement was complete, the sampling arm was opened and remaining sample was wiped from both the upper and lower pedestals using a soft laboratory wipe. Wiping

prevents sample carryover in successive measurements. Results are reported on the computer screen as shown in Figure 3.14.

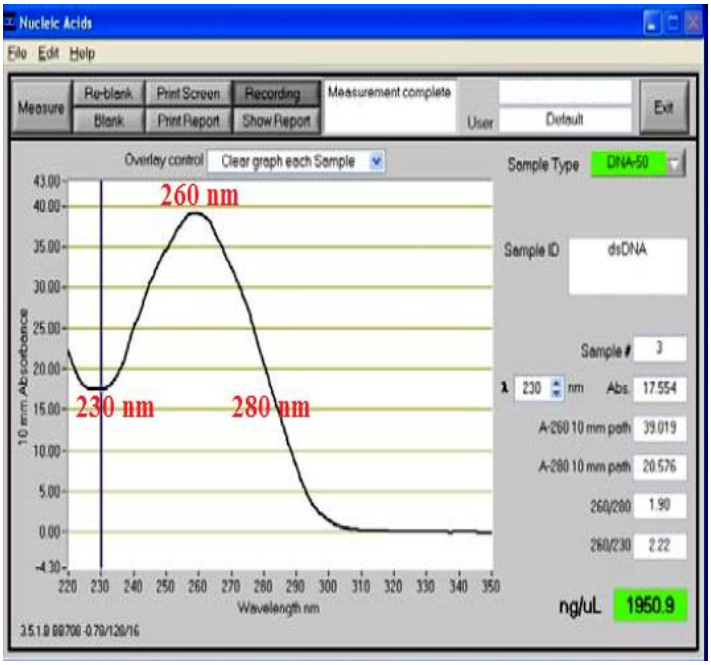


Figure 3.9 NanoDrop reading. *The main (white) window on the screen reports the levels of light absorbance at 3 different wavelengths (230nm, 260nm and 280nm). The software calculates automatically the absorbance ratio 260/280 and 260/230 (right and side). This informs on possible sample contaminations by organic compounds other than DNA. Furthermore, the amount of light with wavelength of 260nm absorbed by the sample provides its DNA concentration.*

The concentration of the DNA was provided in ng/µl. Furthermore, absorbance at A230 A260 A280 was graphically reported with corresponding number on the right side of the screen. This provided information on the concentration of organic compounds, nucleic acids and proteins present in the sample. From these values the software calculates:

- 260/280 ratio: ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ≥1.7 is generally accepted as "pure" for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

- 260/230 ratio: ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure"

nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

In this thesis, DNA concentration was assessed in duplicate in each sample and a novel measure was performed when the difference between the two readings was higher than 10ng/μl. For samples with high concentration of DNA and with persistent variability of the measurements, a further dilution 1:10 was performed as previously described and samples were re-measured.

3.3.1.3 DNA standardization

The concentration of each sample was manually standardized under sterile conditions to 15 ng/μl using the following formula:

$$[WS] \times V_1 = 15\ ng/\mu l \times 50\ \mu l$$

where [WS] is the concentration of the working solution detected using Nanodrop, while V_1 is the volume of the working solution to be used in order to obtain a final DNA concentration of 15 ng/μl in a volume of 50 μl. Therefore, for each sample a volume equivalent to V_1 was added to the corresponding well of a new 96 well plate containing an amount of nuclease and protease free water (Sigma) equivalent to (50 μl – V_1). For those samples with a DNA concentration of the working solution lower than 15 ng/μl (seven in total), the stock solution was used to make the final dilution. At the end of the standardization procedure, the concentration of each sample was checked again with Nanodrop, using the same procedure described above.

3.3.2 Real-Time PCR: principles and result interpretation

Real-time PCR (RT-PCR, also known as quantitative PCR, qPCR) combines PCR chemistry with the ability to detect and continuously monitor the accumulation of reaction products (amplicons) after each amplification cycle. Real-time PCR has the ability to detect the presence and quantity of specific nucleic acid sequences (target nucleic acid), as well as to determine whether sequence variations exist. The amount of amplicons generated during each PCR cycle is directly proportional to the amount of template prior to the start of the run.

3.3.2.1 Principles of PCR amplification and importance of thermal cycles

PCR allows amplifying specific sequences of DNA (templates), which can be single or double-stranded. Two oligonucleotide primers that flank the DNA templates are needed to prime the amplification, together with deossi-Nucleotide-Tri-Phosphate (dNTPs), which are the four nucleotide triphosphates (adenine, guanine, cytosine, thymine), a heat-stable polymerase, and magnesium ions in the buffer. The reaction is performed by consecutive cycles at different temperature. In a standard PCR run, high temperature is applied to separate (melt) the strands of the double helical DNA. The temperature is then lowered to let primers anneal to the template in a highly specific and complementary fashion. Finally, the temperature is set around 72 °C, which is optimum for the polymerase that extends the primers by incorporating the dNTPs. Multiple repetitions of these three thermal cycles allows exponential accumulation of new templates, which will be ultimately ceased due to inhibitors of the polymerase reaction found within the template, reagent limitation and/or accumulation of pyrophosphate molecules (442).

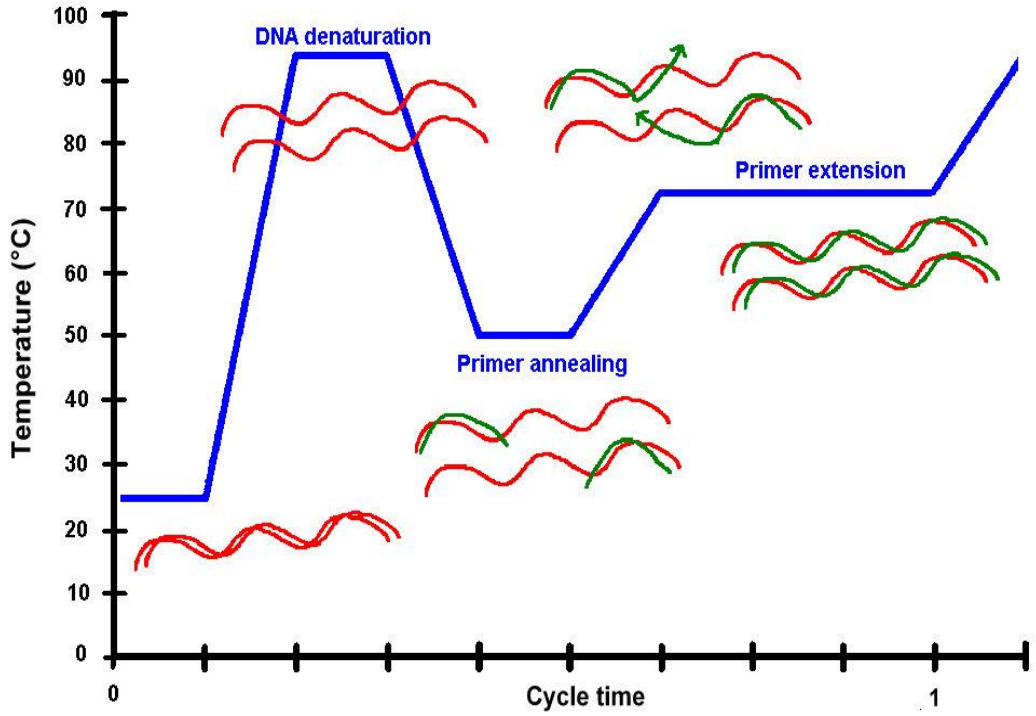


Figure 3.10 The PCR temperature cycle.
Each cycle starts by raising the temperature to about 95°C. This melts the double stranded DNA, making the single stands of DNA available for primer annealing. Following one cycle at low temperature (to let primers anneal), the temperature is set to 72°C to let the polymerase extend the primers.

The melting temperature should be sufficient to fully separate the strands of the template, as a partial separation results in rapid re-annealling when the temperature is dropped, precluding the access of primers to the template. The required temperature and duration of the melting cycle depends on the length and sequence of the template, as well as on the instrument and reaction containers used (443). For instance, with short amplicons and containers with rapid heat transfer (e.g. glass capillaries), it may be sufficient to touch 95 °C and then immediately start the cooling phase. Because the original template is typically much longer than the amplicons dominating the later phase of each PCR run, the first cycle normally requires higher heating temperatures and longer melting times compared to the other cycles (442). The use of elevated elongation temperatures in the following cycles is more important

to melt any secondary structures that may form in the template and may block extension. For example, while amplicons are typically short with limited capability to fold, sequential runs of guanines may fold the template into a tetraplex structure, which is exceedingly stable and cannot be transcribed by the polymerase (444). Similarly, the presence of self-complementary regions between primers, particularly at their 3′-ends, can cause annealing of the primers (known as primer–dimer formation) and leads to premature ceasing of the PCR reaction with a specific amplification signal (445;446). Also the annealing temperature should be carefully defined as it influences the odds of primer-dimer formation, therefore the specificity of the amplification signal. Ideally, the annealing temperature should be only a few degrees below the melting temperature of the two primers (carefully designed to have the same or similar melting temperatures). This would allow them forming stable complexes with the targeted sequences but not with any other sequences, reducing the risk of primer-dimers alignment (445-447).

3.3.2.2 Fluorescent probe used for product monitoring

To follow up the reaction in real time, PCR needs a fluorescent reporter that binds to the product formed and reports its presence by fluorescence intensity (Figure 3.16) in a concentration dependent fashion. A number of probes and dyes can be used as reporters.

Figure 3.11 Reporters used in real-time PCR and their mechanisms. *(A) the molecular beacon, (B) the Taqman probe, (C) the hybridization probes, (D) the LightUp probe, (E) the simple probe, (F) scorpion primer, (G) sequence non-specific dyes (SYBR Green/BOXTO). Adapted from Kubista Mol Aspects Med. 2006;27(2-3):95-125.*

While some of them are sequence specific (Figure 3.16 A-F), those used for LTL assay are non-specific as they intercalate within each DNA molecules presenting a double strand conformation (Figure 3.16 G). In his initial work Higuchi used the common nucleic acid stain ethidium bromide, which becomes fluorescent upon intercalating into DNA (448). However, while classical intercalators interfere with the polymerase reaction, asymmetric cyanine dyes (including SYBR Green I used in LTL

PCR-based assay) do not present this limitation and have become more popular (Figure 3.17) (449;450).

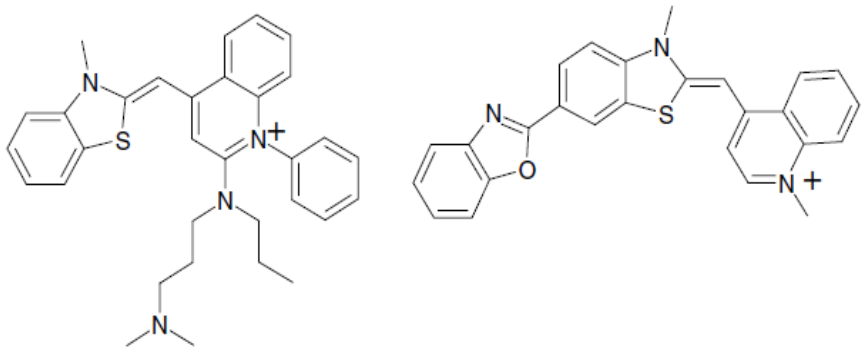


Figure 3.12 The asymmetric cyanine dyes SYBR Green and BOXTO.

These dyes have become popular as their intercalation within the double stand of the DNA does not interfere with the polymerase reaction.Asymmetric cyanines have two aromatic systems containing nitrogen, one of which is positively charged, connected by a methine bridge. These dyes have virtually no fluorescence when they are free in solution due to vibrations engaging both aromatic systems, which convert electronic excitation energy into heat that dissipates to the surrounding solvent. On the other hand the dyes become brightly fluorescent when they bind to double strand DNA, presumably to the minor groove, as rotation around the methine bond is restricted (451). Therefore, the increase in the fluorescence signal is recorded during polymerization, while fluorescence rapidly drops when the DNA is denatured. Fluorescent measurements are then performed at the end of the elongation step of each PCR cycle, allowing direct detection of the increasing amount of amplified DNA. Although the advantage of these fluorescent reporters is that they are relatively cheap as they can detect the increase in any pair of primers for any target, the aspecific nature of their binding gives rise to a fluorescence signal in the presence of any double stranded DNA, including undesired primer–dimer products (450). As

primer–dimer alignment interferes with the formation of specific products because of competition of the two reactions for reagents and leads to erroneous readouts, it is good practice to control for primer–dimer formation after each PCR run. This can be done by melting curve analysis after completing the reaction.

Melting curve analysis is performed by following the change in fluorescence while the temperature is gradually increased. In normal conditions, the fluorescence decreases gradually with increasing temperature because of increased thermal motion which allows for more internal rotation in the bound dye (451). However, when the temperature is reached at which the double stranded DNA strand separates the dye comes off and the fluorescence drops abruptly (452). This temperature, referred to as the melting temperature, *T*m, is easiest determined as the maximum of the negative first derivative of the melting curve. Since primer–dimer products typically are shorter than the targeted product, they melt at a lower temperature and their presence is easily recognized as a second or larger peak in the melting curve analysis (Figure 3.18).

Figure 3.13 Melting curve analysis.
Dye fluorescence drops rapidly when the DNA melts. The melting temperature (Tm) is defined as the inflection point of the melting curve, which is easiest determined as the maximum in the negative 1st derivative of the melting curve. The amplicon produced from the targeted product is typically longer and melts at higher temperature than the primer–dimers. Adapted from Kubista et al. Molecular Aspects of Medicine 2006;27:95-125.

3.3.2.3 Profile of PCR reactions and cycle threshold (Ct)

In PCR methods which use SYBR Green I or, more in general, intercalator molecules to follow the reaction, the plot of fluorescence against the number of PCR cycles displays a typical profile where it is possible to identify three different phases: background, exponential and plateau (453).

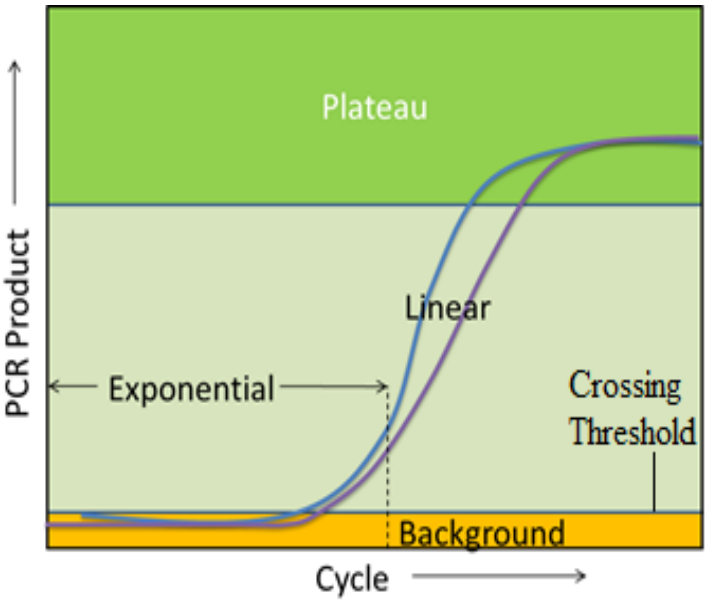


Figure 3.14 Real-time PCR profile for reaction carried with asymetric cyanine dyes. *The 1st phase is hidden under the background fluorescence where an exponential amplification is expected. The 2nd phase is the exponential amplification that can be detected and above the background. Finally, 3th phase or plateau phase is defined as the attenuation in the rate of exponential product accumulation, which is seen concomitantly in later cycles.*

During the initial cycles the relatively low amount of product compared with the high concentration of the fluorescent reporter leaves most of the fluorescent dye suspended in solution and unbounded to the DNA. This results in a weak fluorescent signal, impossible to distinguish from the background (Figure 3.19, background phase). After a few thermal cycles (normally between the 10th and 20th) the accumulation of reaction products increase the number of fluorescent dye conjugated with double strand DNA molecules, leading to emergence of the fluorescence signal from the background. In this phase, fluorescence increases exponentially assuming amplicon doubling at every thermal cycle (Figure 3.19, exponential phase). The exponential phase of amplification normally persists for only few cycles (< 10) as some of the critical components of the reaction run out quickly due their highlighted

expendure. These can be the primers, the reporter, or the dNTPs (454). Also the number of polymerase molecules available may represent the limiting factor, in which case the amplification shifts from an exponential to a linear phase. The end of the exponential growth corresponds with the start of the plateau phase (Figure 3.19), where the fluorescence does not increase any more between different thermal cycles as either there are no more primer or polymerase molecules to produce new products, or there are no more reporter molecules available to bind the new amplicons. As factors that determine the passage from the exponential to the plateau phases equally affect all samples independently from their original concentration, all curves corresponding to the amplification response of each sample are expected to saturate at the same level. Hence, end-point PCR measurements do not provide information about the initial amounts of target molecules that were present in the samples; they only distinguish a positive from a negative sample (455). In the growth phase of the reaction, by contrast, the amplification is not conditioned by the limited availability of the reagents, making it possible to identify difference dependent on the original amount of target gene (TG) in the sample (456). As each template should generate two amplicons, the amplification curves normally present the same slope and appear parallel to each other during this phase. Therefore, the parameters that allow differentiation between samples is not represented by the rate of fluorescence increase, but by number of amplification cycles required for the amplification curves to reach the exponential phase of the reaction (457) (Figure 3.20).

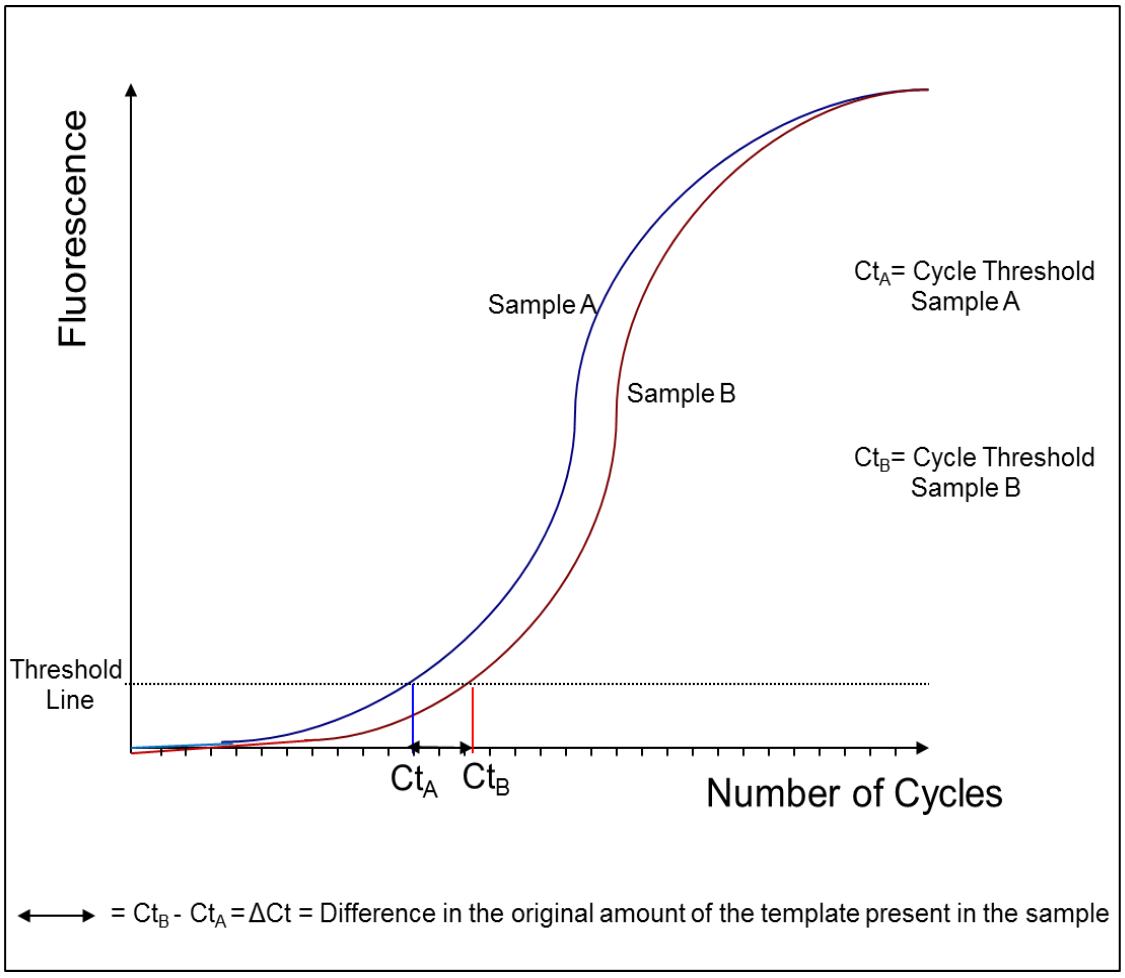


Figure 3.15 Cycle threshold
The number of cycles necessary for each sample to reach the limit of fluorescence detection is directly related to the original amount of DNA or RNA contained in the sample. By comparing the cycle threshold of two samples is therefore possible to establish the difference in the expression of a specific gene or amount of DNA repetitions in the original samples.

As the number of cycles is reported on the x axis of the amplification plot, differences between samples will be visualized as a right or left shift of the curves on this axis. Such a shift depends on the higher or lower number of cycles necessary to reach the exponential phase of the reaction, which is directly related to the original amount of the template present in the sample. Therefore, the definition of the fractional PCR cycle at which the reported fluorescence is greater than the minimal detection level (Ct) allows a precise estimation of the original amount of the template in the sample (458). The minimal level of fluorescence detection (threshold) is

defined using different methods by different instrument software, and most also let the user set it manually. The setting is therefore somewhat arbitrary and it does not affect significantly the differences between Ct values, though it affects the values of the individual Cts. These are also affected by the setting of the instrument (filter, channel, gain, etc.). Hence, one should avoid comparing individual Ct values between experiments, and include one reference per run to which all the other response curves can be related.

3.3.2.4 Quantification and standardization of PCR results

PCR technologies use two major methods to analyse the reaction results: absolute and relative quantification. In both cases a reference is introduced in each run and the final results are expressed as function of this reference.

3.3.2.4.1 *Absolute quantification*

In the absolute quantification method the reference is provided by a standard curve prepared from a dilution series of a control template with known concentration (Figure 3.21)(459).

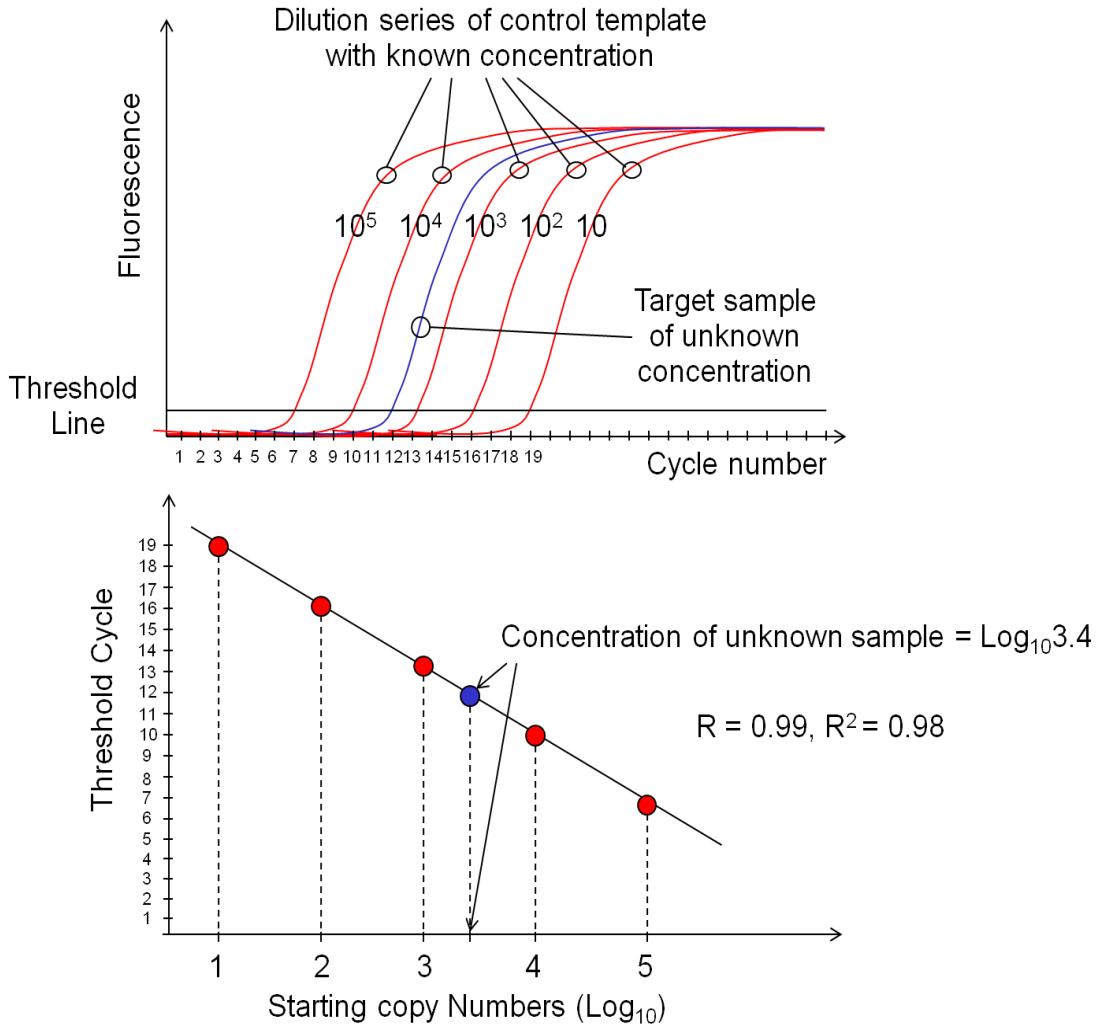


Figure 3.16 Absolute quantification
A serial dilution of a reference sample with known DNA concentration is prepared and run together with the sample of interest. At the end of the reaction, a "calibration" curve is obtained by plotting the cycle threshold vs the original DNA concentrations of the reference sample. Once the calibration curve is available, the concentration of the sample of interest is obtained by overlaying its cycle threshold to the standard curve.

For LTL analysis, the control template is represented by a DNA with known telomere length (e.g. 10.2 kb, Control-DNA high; TeloTAGGG Telomere Length Assay; Roche Applied Science) (71). In order to compare results and ensure similar amplification efficiencies between different runs, the same dilution series of the control template is introduced in each run. Following amplification of the standard dilution series, the standard curve is generated by plotting the log of the initial

template concentration against the Ct generated for each dilution (Figure 3.21) (460). If the aliquoting was accurate and the efficiency of the amplification does not change over the range of template concentrations, the plot of these points should generate a straight line. The comparison of Ct values of the unknown samples with those obtained from the serial dilution of the reference will allow quantification of initial copy numbers (or telomere length) in each sample (460).

Ideally, a standard curve will consist of at least 4 points, and each concentration should be run at least in duplicate (the more points the better). The range of concentrations in the standard curve must cover the entire range of concentrations that will be measured in the assay as conclusions cannot be drawn from samples whose calculated initial quantity exceeds the range of the curve (461). The most important parameter which affects the accuracy of the absolute quantification method is the linearity of the fit between log of the initial template concentrations and the correspondent Ct value (459;460). The linearity is denoted by the R squared value (R^2) and should be very close to 1 (Figure 3.21). A linear standard curve implies that the efficiency of amplification is consistent at all different template concentrations. Conversely, a standard curve that becomes non-linear at very low template concentration suggests that we are approaching the limit of the assay detection. Unknown samples with Ct values that fall within a non-linear section of the standard curve cannot be accurately quantified. The crucial assumption for an accurate quantification is that amplification efficiency of both the reference and sample reactions are very close each other (normally within 5%), and ranging between 90% and 110% (460).

With absolute quantification, therefore, serial dilutions of the target should be run as well and conditions of the reaction should be optimized to ensure the maximal

equivalence of the amplification efficiencies between target sample and control template (462). One hundred percent efficiency implies perfect doubling of amplicon each cycle. If the efficiency is significantly less, this implies the reaction is being slowed, condition which could depend on inhibitors present in the reaction mix or suboptimal primer sets or reaction conditions. By contrast, efficiencies significantly above 100% typically indicate experimental error which can be due to miscalibrated pipettors, contamination or formation of primer-dimer products. In order to exclude possible contamination of the reagents, it is good practice to add at least two no template control (i.e. samples containing only reagent without target and sample template) in each run, which should not produce a quantifiable fluorescence signal.

3.3.2.4.2 *Relative quantification*

In contrast with the absolute quantification method, relative quantification does not express the final results in absolute number of copies or length of the telomere sequences, but as a ratio between the concentration of the target template in the sample of interest and that in a reference sample (463). Therefore, relative quantification does not require a calibration curve or standards with known concentrations.

The levels of target template across multiple samples is obtained by comparing their final Ct values with that obtained from the reference and reporting results as fold-changes relative to this reference. Thus the mentioned methods can be summarized as the ΔCt methods (Figure 3.22) (464;465).

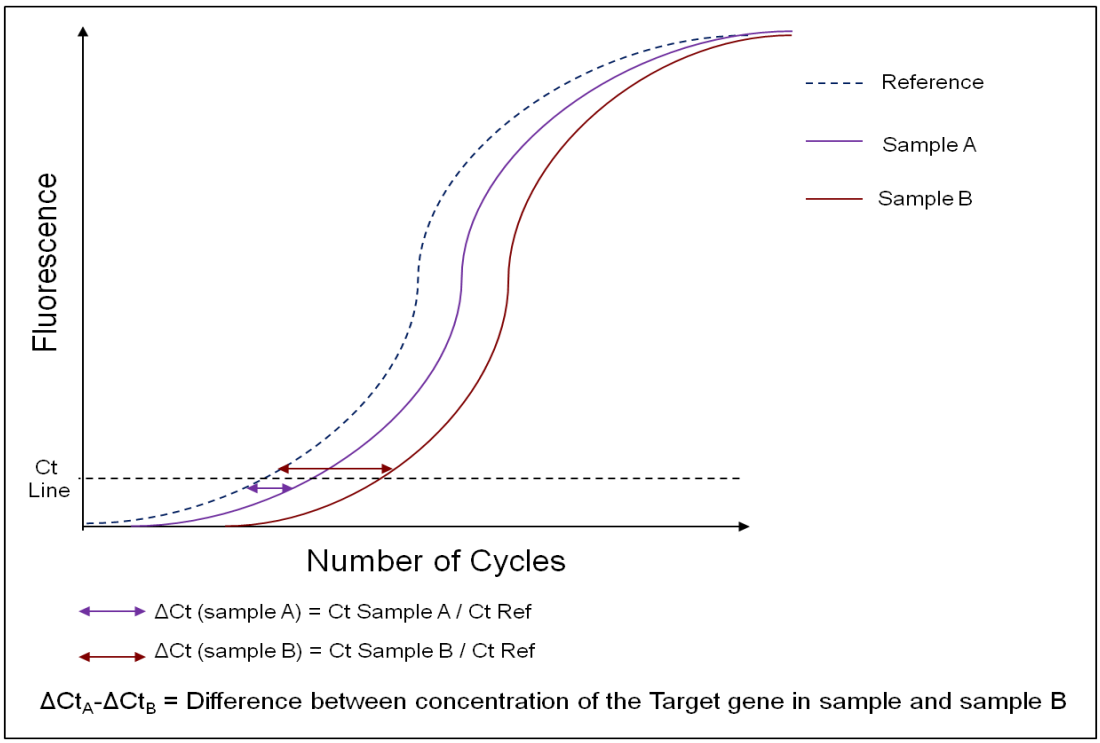


Figure 3.17 ΔCt method of relative quantification.
To compare the concentration of the TG in sample A and B their Ct values are normalised for the Ct value of the TG in a reference sample, which remains the same in different PCR reactions. Therefore, the final concentration of the TG in sample A is provided by the difference between Ct value of TG in A and Ct value of TG in the reference sample (ΔCt_A). Similar calculation is used to establish the concentration of sample B (ΔCt_B). The different concentration of the TG between sample A and B will be then provided by the difference of their Ct values normalised to the reference sample.

Importantly, introducing the same reference in multiple reactions will allow standardization of results between difference PCR runs. But the relative quantification procedure requires a further normalization step which introduces more complexity in the analysis of the final results. To achieve optimal relative expression results, appropriate normalization strategies are required to control for experimental error (466;467), and to ensure identical cycling performance during real-time PCR. These errors can be due to minor differences in the amount of starting DNA/RNA, quality of the DNA/RNA, or difference in PCR amplification efficiencies between samples

(468). Therefore, to ensure identical starting conditions, the relative expression data have to be equilibrated or normalized according to one of the following variables:

- sample size/mass or tissue volume
- total amount of extracted RNA or total amount of genomic DNA
- reference ribosomal RNAs or reference messenger RNAs (mRNA)
- artificial RNA or DNA molecules (= standard material)

Normally, a “housekeeping” gene (HKG) is selected for this further normalization (Figure 3.23). As the HKG expression is expressed at constant levels between different samples or present in single copy within the genome, variation in its amount reflects variations in the concentration of the original RNA/DNA.

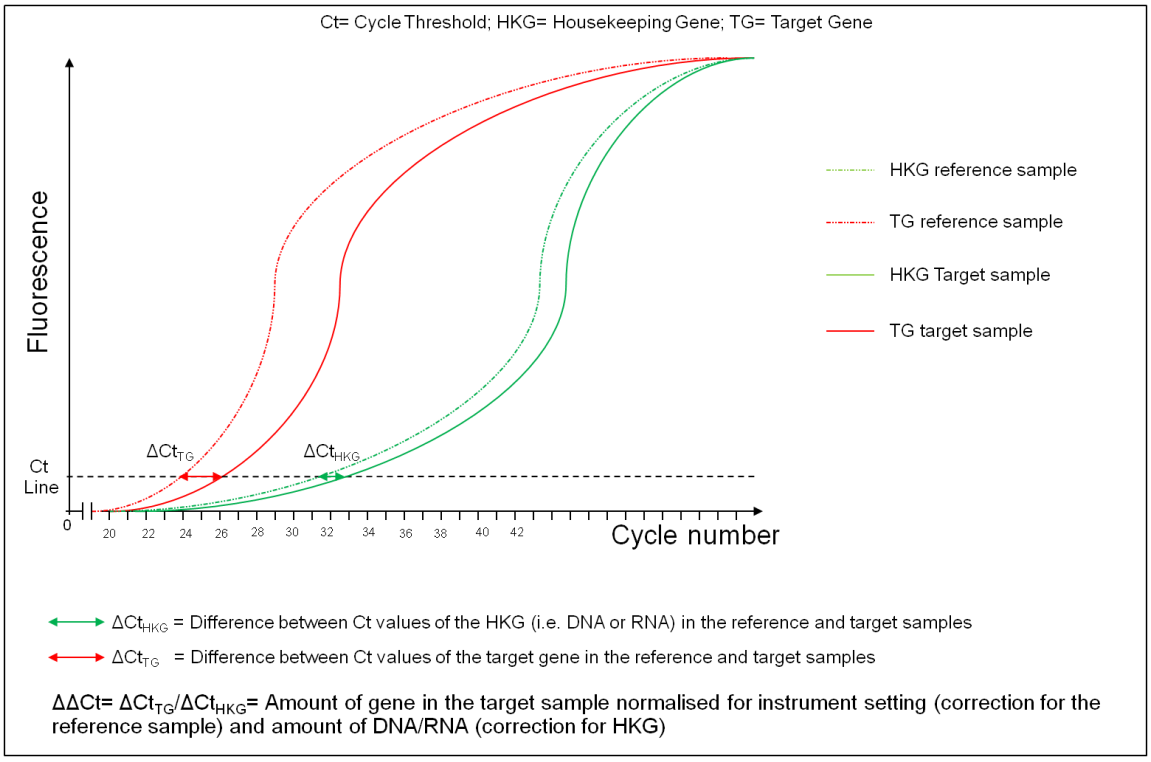


Figure 3.18 ΔΔCt Method of relative quantification
This method introduces a further correction which normalises results for the concentration of an housekeeping gene in the target samples as well as in the reference sample. As the HKG should be expressed at constant levels between different samples or present in single copy within the genome, changes in the concentration of the HKG gene will inform on possible differences in the amount of starting DNA/RNA, quality of the DNA/RNA, or PCR amplification efficiencies between samples within the same run or between different PCR runs.

Selection of an appropriate HKG represents one of the most sensitive parts in the set-up of a PCR experiment, particularly when we want to explore different levels of gene expression between different tissues or in different experimental conditions. It is expected that modest changes in the HKG expression between different tissues or before and after any specific treatment can dramatically affect the quality of the normalization and, subsequently, the final results of the experiment (Figure 3.23).

One example could highlight the relevance of this factor.

Example

Aim - to evaluate the difference in expression of a TG between two samples (A and B) assayed in 2 different days.

Step 1 - Obtain the Ct values of the reference gene (Ref) as well as of the TG in the sample A (day 1) and B (day 2) (Figure 3.24A.A and 3.24.A.B, split in two different pages).

A) Sample A (Day 1) **B) Sample B (Day 2)**

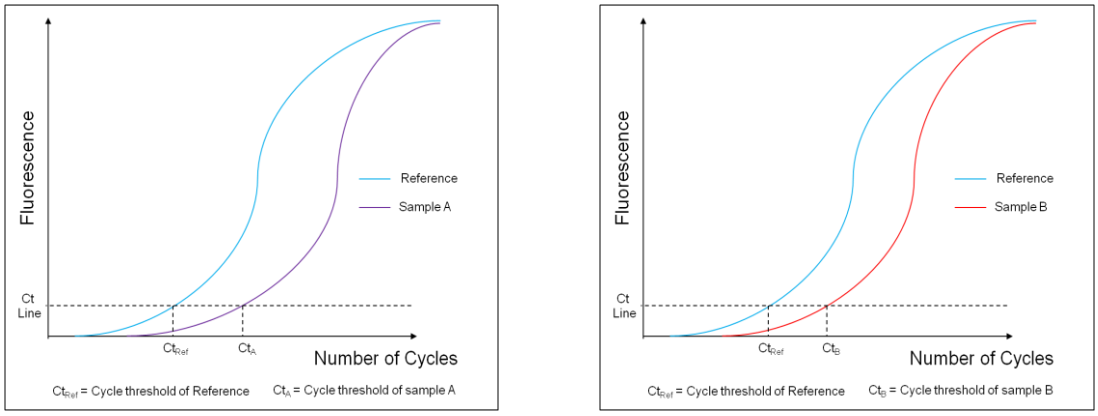


Figure 3.19A Establshing Ct values.
Each PCR run will provide the Ct values of the TG in sample A (A) and B (B) as well as that of the reference sample.

Step 2 – Normalise TG for Ref.

As Ref is the same sample in both reactions, its final Ct value should be the same in the run including sample A and B. Therefore, correction for Ref will allow adjustment for possible difference in the PCR instrument setting between the pre- and post-treatment runs. Moreover, it will inform on how many folds the expression of the gene of interest differs between sample A and sample B compared to the constant reference (Figure 3.24B).

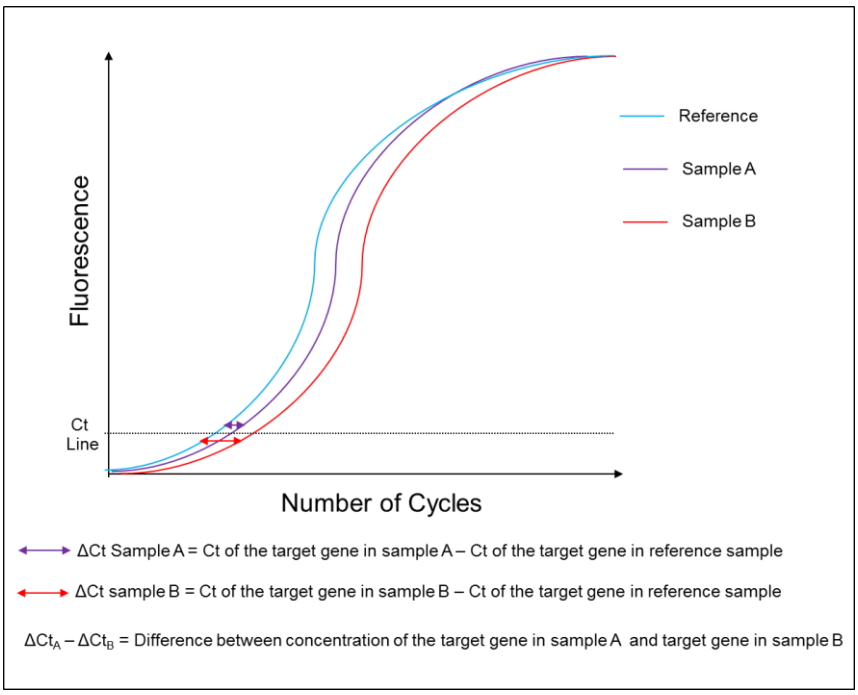


Figure 3.24B Calculating the ΔCt for sample A and B.

Following normalization of the Ct values of the TG in sample A and B, the difference between samples of the TG is calculated with the ΔCt method.

Step 3 - Apply a further correction for the levels of expression of the HKG.

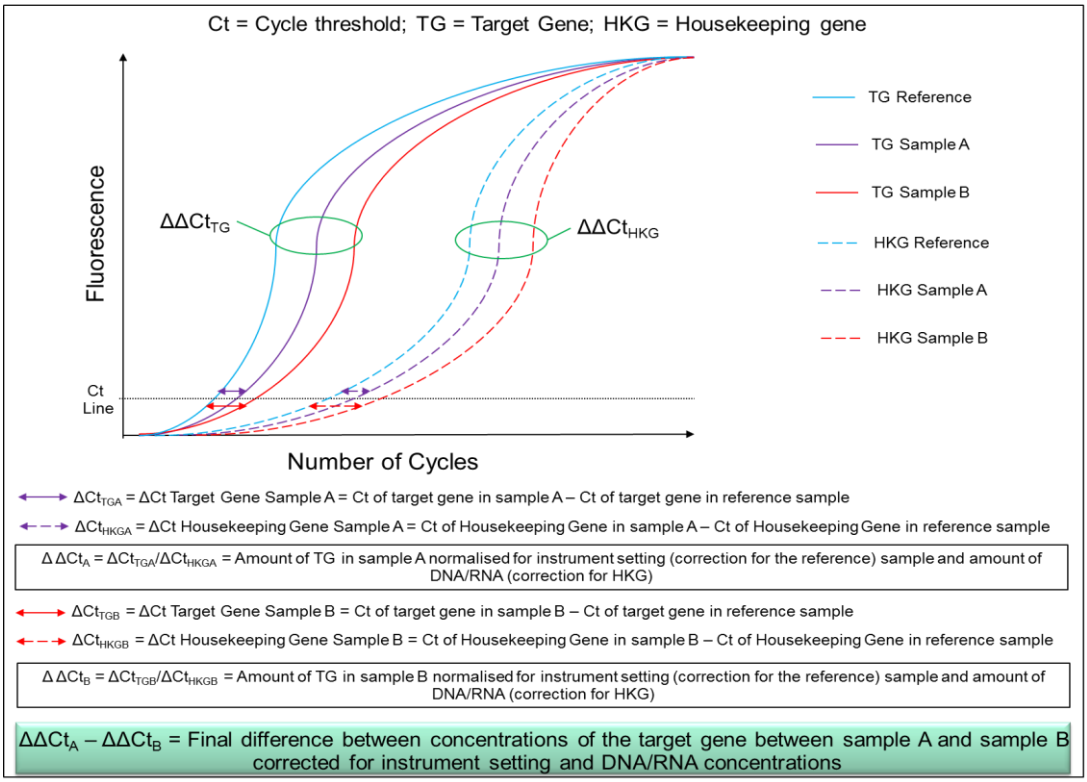


Figure 3.24C From ΔCt to ΔΔCt.

The same samples A and B are run a second time together with the reference sample to establish the concentration of the selected HKG. Normalization of the ΔCt for the TG in sample A and B with the ΔCt for the HKG in the same samples will allow correction for possible difference in amplification efficiency or original DNA concentration between sample A and B.

Assuming levels of expression of the HKG are not affected by treatment, a possible difference of the HKG levels following treatment will suggest that there was a change in one of the following steps between the two time points:

a) the quality of the extracted mRNA

b) the efficiency of the reverse transcription or

c) the total cDNA concentration

As each of these factors could equally affect levels of TG expression, correction of TG for HKG will allow correction of the final results for all these possible sources of variability.

In the LTL PCR-based assay, the most widely used HKG are: β-globin, GAPDH and 36B4 (71;82;83). These genes are selected not because their levels of expression are constant in different experimental conditions, but because they are present in single copy in the genome. Therefore, their Ct values at the end of a PCR run provides information on the amount of genomes (e.g. the original amount of cells) that were present in the original sample. Consequently, normalization of the telomere results for these genes allows quantification of the average amount of telomere repetition per copy of genome (i.e. per cell) in the sample.

Selection of one specific HKG to be used in the PCR assay depends on the level of experience of each laboratory. However, the amplification efficiency of the HKG reaction should always completely overlap with that of the telomere reaction. Selection of primers, the PCR setting and the concentrations of reagents should be optimized to achieve this goal. The amplification efficiency of the HKG and telomere reactions is commonly checked by standard curve analysis (71;82;83). As previously mentioned for the absolute quantification, serial dilutions of a randomly selected

sample are prepared and the telomere and HKG reactions are performed. Following amplification, a standard curve for the telomere and HKG is generated by plotting the log of the initial template concentration against the Ct generated for each dilution. The value of R^2 will inform on the amplification efficiency of both reactions. This will allow comparison of the amplification efficiencies between HKG and telomere reactions, with optimisation of the primers and PCR setting. Furthermore, it will inform on whether the same amplification efficiency is maintained at different concentrations of the templates during the HKG and telomere reaction.

3.3.2.4.3 *Mathematical models of relative quantification*

The relative expression of a target template in relation to the reference gene, can be calculated using the **comparative threshold method**, also known 'delta delta Ct' (ΔΔCt) values (465). With this method, the amount of TG is estimated by calculating the number of folds it differs from the reference gene and normalizing this ratio for the amount of HKG in the sample. Therefore the formula used to obtain the final results is expressed as:

$$2^{-\Delta\Delta Ct}$$

where

$$\Delta\Delta Ct = \Delta Ct(\text{sample}) - \Delta Ct(\text{reference})$$

and ΔCt is the difference between Ct of the TG and that of the HKG in both target and reference samples (465;469)(Figure 3.24C).

The number "2" of the formula relates to the assumption that, at each cycles, the amount of product doubles the original amount of the template. Subsequently, to

obtain accurate results with this formula it is crucial that the amplification efficiency of the target (e.g. telomere) and HKG reactions are the same and constant between different samples (possibly above 90%) (465). However, several conditions may cause significant variations in the PCR efficiency between different samples, possibly resulting in inaccurate estimation of gene expression levels with this method of quantification. The error can be calculated as a function of the PCR efficiency and the cycle number according to the formula:

$$\text{Error (\%)} = [(2^n/(1+E)^n) \times 100)] - 100$$

where "E" represents the efficiency of PCR and n the cycle number. Therefore, if the PCR efficiency is only 0.9 instead of 1.0, the resulting error at a threshold cycle of 25 will be 261%. The calculated expression level will be 3.6-fold less than the actual value. Thus, the ΔΔCt method should only be chosen if we are confident that the PCR efficiency of the TG and HKG are the same and do not change between samples, or if the difference in expression levels is sufficiently high to tolerate the resulting error.

Changing PCR efficiencies between samples can result from several factors including different concentrations of PCR inhibitors or enhancers as well as variations in the RNA/DNA pattern extracted. As the efficiency evaluation is an essential marker and the correction is necessary for accurate real-time gene quantification (470-473), researchers have introduced the amplification efficiency parameter in the "delta delta Ct" or comparative threshold cycle method.

The efficiency corrected calculation model, based on one sample (474) is expressed as:

$$\text{Ratio} = \frac{(E_{TG})^{\Delta Ct\ TG\ (reference - sample)}}{(E_{HKG})^{\Delta Ct\ Ref\ (reference - sample)}}$$

while the efficiency corrected calculation model based on multiple samples is reported as (462):

$$\text{Ratio} = \frac{(E_{TG})^{\Delta Ct\ TG\ (MEAN\ reference - MEAN\ sample)}}{(E_{HKG})^{\Delta Ct\ HKG\ (MEAN\ reference - MEAN\ sample)}}$$

The PCR amplification efficiency can be estimated by the standard curve method or by mathematical calculations which extrapolates this information from the slope of the exponential phase of the reaction. With the standard curve approach, efficiency can be determined by preparing a regression plot reporting on the *x* axis a serial dilution of the unknown and reference samples and on the *y* axis the corresponding Ct values. From this plot the amplification efficiency is provided by the following equation (453;475):

$$E = 10^{[-1/slope]}$$

But preparing a dilution series of each target sample is laborious, costly (as it increases the amount of reagents and templates to be used in each PCR reaction), and takes time. To overcome this limitation, a single dilution series composed of a pool of all unknown RNA/DNA samples can be added to each PCR run. As this standard curve is obtained from a mixture of all samples run in the PCR reaction, the resulting "E" value should partially reflect all possible 'positive and negative impacts' on kinetic PCR efficiency. Although this solution can reduce time and cost of the reagents, it is still necessary to introduce a standard curve in each PCR reaction to estimate the amplification efficiency of each run and account for its possible changes between different runs.

For this reason, a novel and more reliable method to calculate the amplification efficiency, known as the **comparative quantification method**, was proposed by the manufacturers of the RotorGene, a Real Time Theramal Cycler (RotorGene 5.0 software, Corbett Research, Australia). It is based on the

measurement of the relative fluorescence increase in the exponential phase of the PCR reaction and allows automatic determination of the real-time PCR efficiency sample-by-sample, obviating the need for standard curves to generate efficiencies and controlling for efficiency differences between reactions (475). For each sample, a linear regression plot is drawn from at least four data points of the exponential amplification phase. The slope of the regression line represents the PCR efficiency for that sample (476;477). However, the investigator had to decide which fluorescence data of the exponential phase to include in the analysis and which to omit. If fitting is performed by eyes, its final result could be highly operator dependent as the growth in fluorescence rises quickly in exponential phase and it remains difficult to identify the exact point at which the fluorescence emerges from the background (the Ct value). Therefore, the lower threshold point of the fitting curve may inadvertently be set outside the exponential phase of product amplification, drastically influencing the slope of the curve (Figure 3.25).

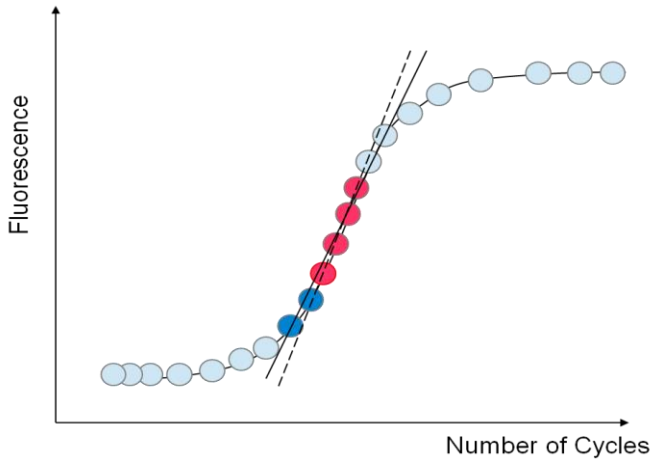


Figure 3.20 Estimation of amplification efficiency from the exponential phase of the amplification. *The arbitrary estimation of the first point to include in the fitting line used to calculate the amplification efficiency can result in significant changes in the estimated amplification efficiency. In this example, the use of blue points rather than the red point results completely different slopes curves, which correspond to two different values of amplification efficiency.*

To obviate this limitation, it was decided to use the second derivative of raw fluorescence values to help calculate the point at which the exponential phase of amplification begins (472;475). This point, termed the Take-Off Point (TOP), is equivalent to the Ct value and therefore used also to calculate the difference in the template amount between the reference and the target samples. In the Rotor-Gene software package the TOP is arbitrarily described as the point 80% below the second derivative plot peak (478)(Figure 3.26).

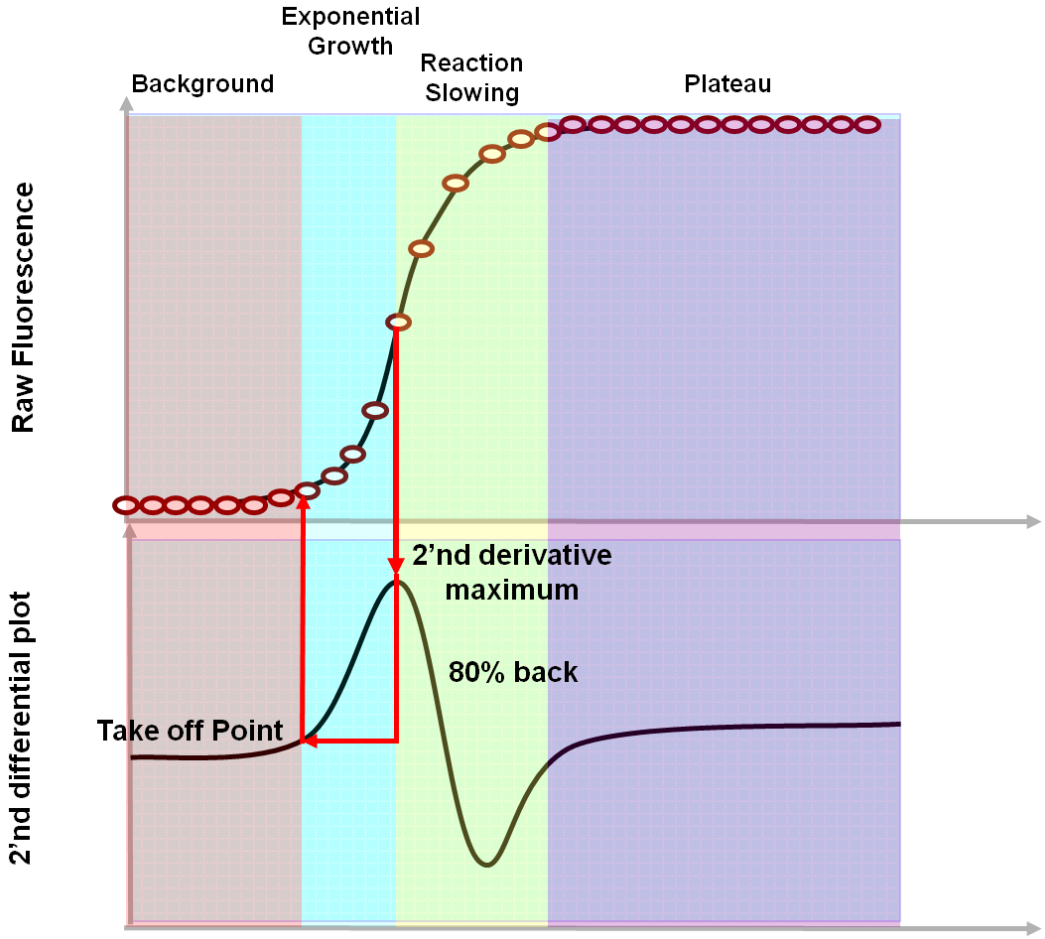


Figure 3.21 Comparative quantification method.
From the second derivative of the raw fluorescence data it is possible to estimate the maximum exponential growth of the product (peak of the parabola). The TOP, which is 80% below of the peak level, represents the point at which the exponential phase of the amplification begins. It is used to define the Ct of each sample and it marks the first of the four consecutive points used to calculate the amplification efficiency during the exponential phase of the reaction.

The first four fluorescent readings following the TOP in the second derivate plot are transposed into the original amplification plot and used to calculate the amplification efficiency during the exponential phase (478). In this part of the PCR reaction, the increase in fluorescence (F) is represented by the exponential growth model:

$$F_{n+1} = F_n * (E)$$

where "n" is the cycle number and "E" is the measure of the efficiency of the reaction (amplification value). Therefore, by rearranging the formula it is possible to calculate an observed amplification (E_n) of each point included in the exponential phase of a reaction:

$$(E_n) = F_{n+1}/F_n.$$

Averaging the amplification over the first four readings following the TOP produces the amplification value of the sample (E_s). Outlier amplifications are removed to account for noise in background fluorescence (478).

In conclusion, using the comparative quantification method, the amount of gene product in any given sample relative to a designated reference sample is calculated by rearranging the comparative threshold cycle formula as follow:

$$\text{Relative Quantity (RQ)} = (E_s)^{\Delta\text{TOP (Reference – Sample)}}.$$

The RQ values for the TG are then transformed into a ratio of the HKG values for each individual sample:

$$RQ = \frac{(E_{TG})^{\Delta\text{TOP TG (Reference – Sample)}}}{(E_{HKG})^{\Delta\text{TOP HKG (Reference – Sample)}}}$$

3.3.2.5 Protocol of LTL Assay

In this PhD, LTL was measured with a PCR based assay in accordance with the original technique reported by Cawthon (82) and adapted for the Rotor-Gene 6000 machine (479), unless otherwise specified. In each sample, the quantity of telomere repeats and the amount of genome were determined in comparison to a reference sample in two different quantitative PCR reactions. The single copy gene (SCG) 36B4, which encodes acidic ribosomal phosphoprotein PO and is located on chromosome 12 (480), was used as HKG to inform on the amount of genome present in each sample. A null template control (NTC) sample was added to each SCG and telomere reactions to check for non-specific amplification (i.e. contamination of the reagents, not-specific product formation). Similarly, a reference sample, obtained by mixture of randomly selected clinical samples from a study previously conducted in our lab, was used to normalize SCG and telomere results and check consistency of the PCR reaction conditions between different runs.

The primers used for the telomere and the SCG amplification were as in Cawthon's report (82):

telomere forward: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT

telomere reverse: TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA

SCG forward: CAGCAAGTGGGAAGGTGTAATCC

SCG reverse: CCCATTCTATCATCAACGGGTACAA.

Telomere primer pairs were designed by Cawthon to specifically amplify the telomeric hexamer repeats without generating primer-dimer products (82).

DNA polymerase can extend each primer from its 3′-end when it is hybridized to telomere hexamer repeats but not when it is hybridized to the other primer. Six bases on the 5′-end of each primer are not complementary (base unpaired) with the

telomere sequence, even when the rest of the primer is optimally hybridized. The complements of these 5′-sequences are generated at the 3′-ends of all products that are completed in each cycle of the PCR, thereby blocking those 3′-ends from initiating DNA synthesis in the middle of telomere amplification products in subsequent cycles (Figure 3.27). SCG primer design was similar to those reported in other PCR applications where the 36B4 gene was used as the HKG.

Figure 3.22 Telomere primer design.
(A) *Annealing of primers to genomic DNA. The telomere forward primer can hybridize to any available partially complementary 31 bp stretches along the strand of telomeric DNA oriented 5′→3′ toward the centromere. The telomere reverse primer can hybridize to any partially complementary 33 bp stretch along the strand oriented 5′→3′ toward the end of the chromosome. In both of these primer–template hybridizations, every sixth base is mismatched, however, the last five bases at the 3′-end of the primers are perfectly matched to complementary bases in the template. Addition of bases by DNA polymerase begins at the 3′-ends of the annealed primers and proceeds in the direction of the large arrows.* ***(B)*** *Annealing of primers to each other. The strongest possible hybridizations of the primers to each other involve a repeated pattern of six bases containing four consecutive paired bases followed by two mismatched bases, an example of which is shown here. The 3′-terminal base of each primer cannot form a stable base pair with the base opposite it, thereby blocking addition of bases by DNA polymerase. Adapted from Cawthon RM.* Nucleic Acids Research. *2002;30(10): e47.*

Reaction conditions and concentration of the primers were similar to those described by Cawthon (479). In the telomere PCR, primer concentrations were 135/900nM (forward/reverse) and the cycling profile: 95°C incubation for 10min, followed by 22 cycles of 95°C for 15s and 58°C for 120s. In the SCG PCR, primer concentrations were 300/500nM and the cycling profile: 95°C incubation for 10min, followed by 30 cycles of 95°C for 15s and 58°C for 60s. For both the telomere and the SCG PCR the final reaction volume was 25µl consisting of 1× SYBR Green, 1× qPCR mix (2× SensiMix NoRef DNA kit, Quantace, London, UK), 30ng of template, and the respective primer concentrations. The specificity of all telomere and SCG amplifications was determined at the end of every run by melting curve analysis. The presence of a single peak confirmed the amplification of a single and specific template.

The comparative quantification method was used to generate the final results. At the end of the telomere reaction the number of telomere repeats in each sample was calculated using the formula:

$$\text{Telomere (T)} = (E_{tel})^{\Delta\text{TOP tel (Reference – Sample)}}$$

while the amount of genome presents in the same sample was estimated with the SCG reaction according with the formula:

$$\text{SCG (S)} = (E_{SCG})^{\Delta\text{TOP SCG (Reference – Sample)}}$$

The final results were obtained from the ratio between telomere and single copy gene (T/S) which represents the average number of telomere repeats per copy of

genome in the sample. Every sample (including reference and NTC) was run in duplicate and the average of the two measurements was used for statistical analysis. If at the end of the telomere or SCG reaction one sample presented a standard deviation > 0.2 between duplicate, both reactions were repeated for that specific sample. Similarly, when the NTC identified non-specific amplifications or the standard deviation between duplicates of the reference sample was >0.2, the telomere and SCG reactions were repeated for all samples included in the run.

Validation of this PCR assay was previously performed by measuring 32 DNA samples of subjects aged 24 to 54years from the Cardiovascular Sciences Department, Leicester University DNA bank with both the standard Southern Blot (providing the size of TRF) and the currently described PCR-based method (479). The good correlation between relative T/S ratios measured by quantitative PCR and relative TRF lengths measured by the traditional Southern blot approach confirmed that the new PCR method does indeed measure relative telomere lengths.

The coefficient of variation for each set of samples analysed in this thesis are reported in the single studies.

3.4 Colorimetric assays

In study 2, a symmetrical sandwich ELISA was used to determine the serum levels of insulin while lypopolisaccaride (LPS) was measured using a Limulus Amebocyte Lysate (LAL) colorimetric assay.

3.4.1 Insulin assay

ELISA's combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies coupled to an easily assayed enzyme (481). One of the most useful of these immunoassays is the two antibody "direct sandwich"

ELISA, which allows measuring the concentration of a specific compound in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, it can be used to determine the absolute amount of antigen in an unknown sample. The "direct sandwich" ELISA requires two antibodies which bind epitopes on the antigen (compound measured) that do not overlap. This can be accomplished with either two monoclonal antibodies that recognise discreet sites on the antigen or one batch of affinity-purified polyclonal antibodies (482).

To utilize this assay, one antibody (the *"capture"* or *first* antibody) is purified and bound to a solid phase typically attached to the bottom of a microtitration well. The unknown sample is added to the well and left to incubate for few minutes/hours. During incubation, the target antigen in the sample reacts and complexes with the antibody bound to the solid phase. A second antibody (the *"detection"* antibody) is added during or at the end of this incubation period. This second antibody is conjugated with an enzyme and reacts with a separate antigenic determinant of the target molecule, thus completing the "sandwich". Following the incubation of the secondary antibody, various washing steps allow removing the unbound secondary antibody. To determine the amount of target antigen complexed between the two antibodies, the substrate specific for the enzyme labelled to the detection antibody is added to the reaction. Ideally the enzyme substrates should be stable, safe and inexpensive. Substrates are colourless and are converted by the enzyme, to a coloured product, e.g. blue. The final addition of an alkaline phosphatase converts the blue colour into yellow, making it possible the estimation of the optical density of the solution by absorbance (Figure3.28).

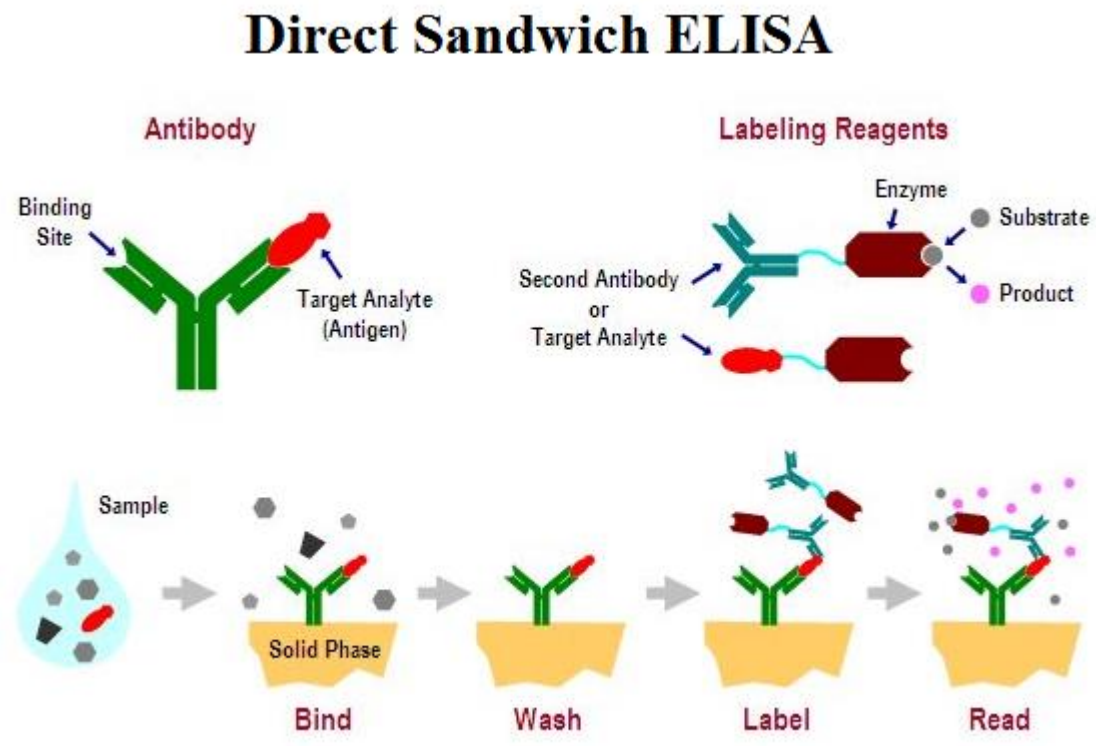


Figure 3.23 Direct Sandwich ELISA
The sample is added to a well coated with specific antibodies which bind the antigen of interest. Antigen is "captured" by coating antibodies during incubation. The unbound antigen is washed away and enzyme conjugated second antibodies directed against a different epitope of the antigen are added. Binding of the second antibody during incubation completes the "direct sandwich". Following a second washing step necessary to remove the unbounded second antibody, a colourless substrate is added and is converted to coloured product by the antibody-bound enzyme. The colour is then quantified in a spectrophotometer.

<u>*Assay:*</u> All samples were thawed in a 4°C fridge overnight. Before proceeding with the assay the enzyme conjugated and wash solution provided with the kit were properly diluted as follow. 1 volume (1ml) of the 11x solution containing peroxidase conjugated mouse monoclonal anti-insulin antibodies (enzyme conjugated solution) was diluted in 10 volumes (10ml) of the enzyme conjugated buffer to obtain the correct amount of "working enzyme solution" necessary for a 96 well plate. Similarly, 1 volume (1ml) of the 21x concentrated washing buffer was diluted in 20 volumes (20ml) of redistilled water to obtain the working wash solution enough for a

96 well plate. Six calibrator solutions containing serial dilutions and known concentrations of recombinant human insulin were added to each well in order to obtain a calibrator curve. This curve allows understanding the concentrations of insulin which correspond to different levels of absorbance. Therefore, by comparing the value of absorbance of each sample with the values obtained from this calibrator curve it is possible to obtain the absolute values of insulin concentration in the interrogated sample.

25 µl of unknown samples and of each calibrator solutions were added in duplicate to a 96 well plate coated with mouse anti-insulin monoclonal antibodies. 100 µl of working enzyme solution (containing the secondary mouse monoclonal antibodies specific for a different epitope on the insulin molecule and conjugated with the peroxidase enzyme) was added to each well followed by incubation on a plate shaker (700-900 rpm) for 1 hour at room temperature (18-25°C). During this incubation the insulin molecules of the sample complex with the antibodies bound to the microplate and with the secondary antibody dispersed in the working enzyme solution. After incubation, the reaction volume was discarded by inverting the microplate over a sink. 350 µl wash solution was added to each well to remove the unbounded secondary antibodies. The wash solution was discarded by firmly tap several times against absorbent paper to remove the excess of liquid. This washing step was repeated 5 times. Once all the excess secondary antibody was removed, 200 µl of solution containing 3,3'5,5'- tetramethylbenzidine base (TMB) was added into each well to detect the bounded secondary antibody. The TMB solution was left in each well for 15 minutes at room temperature to allow complete reaction of all bounded enzymes with the detection solution. During this reaction, samples acquired a blue colour, the intensity of which is directly related with the amount of enzyme

(and secondary antibody) present in the each well. At the end of the incubation, 50 µl of a solution containing 0.5 M of H_2SO_4 was added to the wells to stop the enzymatic reaction. The plate was placed on a shaker for 5 seconds to ensure complete mixing. The addition of the stop solution shifts the colour from blue to yellow in a concentration dependent fashion. Plates were then read at an optical density of 450nm using a Dynatech MRX plate reader (Acterna, Aldermaston, UK) with the standards plotted as a sigmoid curve (Revelation software). Samples with a final insulin concentration ≥200 mU/l were diluted 1:10 and the assay was repeated.

3.4.2 LPS assay

The use of LAL for the detection of endotoxin evolved from the observation by Bang that a Gram-negative infection of Limulus polyphemus, the horseshoe crab, resulted in fatal intra-vascular coagulation, even if the bacteria were killed (483). Levin and Bang later demonstrated that this clotting was the result of a reaction between endotoxin and a clottable protein in the circulating blood cells (amoebocytes) of Limulus (484;485). Following the development of a suitable anticoagulant for Limulus blood, they prepared a lysate from washed amebocytes which was an extremely sensitive indicator of the presence of endotoxin. This led to the production and commercialisation of the LAL assay for endotoxin detection in biological samples, which was approved with guidelines by the FDA in the 1987 (486).

According with this assay, Gram-negative bacterial endotoxin present in the samples catalyzes the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the splitting of pNA from the synthetic colourless substrate Ac-lle-Glu-Ala-Arg-pNA, producing a yellow colour.

$$\text{Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Active Enzyme}$$

$$\text{Ac-lle-Glu-Ala-Arg-pNA} + H_2O \xrightarrow{\text{Active enzyme}} \text{Ac-lle-Glu-Ala-Arg} + \text{pNA}$$

This is measured photometrically at 405-410 nm, after the reaction is stopped with stop reagent. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1-1.0 EU/ml range. The concentration of endotoxin in a sample is calculated from the absorbance values of solutions containing known amounts of endotoxin standard.

Assay: All manual steps of the LPS assay were performed under a sterile hood, using endotoxin-free water (LAL Reagent Water, provided with the kit) and sterile pipettes in order to minimise the risk of endotoxin contamination. The test was performed using 96 well plates which were pre-equilibrated at 37°C in a heating block adapter. Meanwhile, four dilutions of the lyophilized endotoxin at a standardized concentration provided with the kit were prepared for the determination of the standard curve. The lyophilized endotoxin was reconstituted by adding 1 ml of pre-warmed LAL Reagent Water. As the concentration of lyophilized endotoxin may vary from 15-40 EU depending on the lot number, the actual concentration of endotoxin after this step corresponded to the value stated on the certificate analysis. For example, if the value reported on the vial of lyophilized endotoxin was 20 EU, after reconstitution with 1 ml of LAL Reagent Water, the concentration of the endotoxin stock was 20 EU/ml. The first dilution containing 1.0 EU/ml was prepared in a suitable container by diluting 0.1 ml of the endotoxin stock solution with (κ-1)/10 ml of LAL Reagent Water, where 'κ' equals to the concentration of endotoxin in the stock solution. For example, if κ=20 EU/ml, then 0.1ml of κ was diluted in 1.9ml of LAL Reagent Water, (20-1)/10ml, to obtain the first dilution containing 1.0 EU/ml of

endotoxin. Following, 0.5 ml of this 1 EU/ml solution were transferred in a new container and 0.5 ml of LAL Reagent Water was added to obtain the second endotoxin dilution (0.5 EU/ml). Similarly, 0.5 ml of the 1 EU/ml solution was transferred in a vial containing 1.5 ml of LAL Reagent Water to obtain the third endotoxin dilution (0.25 EU/ml). Finally, 0.1 ml of the 1 EU/ml solution was transferred in a vial containing 0.9 ml of LAL Reagent Water to obtain the last dilution of endotoxin (0.1 EU/ml). Once all dilutions were prepared, 50 µl of the target sample or of the dilution series were added in duplicate into the appropriate microplate well. Each series contained a blank well control, where the samples or dilutions were substituted with 50 µl of LAL Reagent Water.

At time T=0, 50µl of LAL were added to the first column of microplate wells using a multi-channel pipettor and reagent reservoir. The sequence of column as the LAL is added was taken into account in order to be consistent in the order of reagent addition from row to row, and in the rate of pipetting. Once the LAL was dispensed into all microplate wells containing samples (or standards), the microplate was briefly removed from the heating block adapter and repeatedly tapped on its the sides to facilitate mixing. The plate was returned to the heating block adapter and covered. Following 10 minutes, 100 µl of substrate solution (prewarmed to 37°C ± 1°C) were added. The substrate solution was pipetted following the same order of the LAL and trying to maintain a consistent pipetting rate. Once completed the addition of the substrate solution, the microplate was briefly removed from the heating block adapter and repeatedly tapped to facilitate mixing. The plate was then returned to the heating block adapter and covered. The addition of the substrate caused a progressive change in the colour of the solution, which became yellow. Following 16 minutes, 50µl of stop reagent was added maintaining the same pipetting order as in of LAL and

substrate addition. The plate was again removed from the heating block and tapped to allow proper mixing. The absorbance of each microplate well was read at 405-410nm, using distilled water to adjust the photometer to zero absorbance.

Serum inhibition of LPS detection with the LAL tests is possible (487-494), particularly when using the colorimetric assay. This inhibition results in a lower, final Δ absorbance, indicating lower levels of endotoxin than what may actually be present in the test sample. Therefore, the lack of product inhibition should always be tested. In this thesis, the possible inhibition of the LAL reaction was determined for each sample using the method reported in the kit instruction. To verify the lack of product inhibition, an arbitrary dilution (1:5) of test sample was spiked with a known amount of endotoxin (0.4 EU/ml). To prepare a 0.4 EU/ml endotoxin solution in the diluted sample, the 1.0 EU/ml solution was diluted 1:2.5 using the diluted sample as the diluent. The spiked solution was assayed along with the unspiked samples and their respective endotoxin concentrations were determined. If the difference between these two calculated endotoxin values was not equal to the known concentration of the spike ± 25%, this suggested the presence of inhibitory factors to the LAL reaction in the sample and the sample was further diluted before the LPS assay.

Using this method, the coefficient of variation obtained from a randomly selected set of 30 samples of study 2 which were run in two different days was 3.27%.

3.5 Statistics

All data are expressed as mean±SD unless otherwise stated. A number of parametric and non-parametric tests were used in this thesis. Parametric tests were used whenever possible (i.e when the data were normally distributed, or could be easily transformed to normality). If the data could not be easily transformed, non-

parametric tests were performed. Normality of the data was assessed by plotting histograms and applying the Kolmogorov-Smirnov test of normality. Stata version 8 and SPSS version 10 analysis packages were used for the analysis of the data.

Parametric tests used were independent t-test, paired t-test and one-way analysis of variance (ANOVA). In the case of studies where the means of more than two variables were compared by t-tests a Bonferroni correction was applied. This states that when 3 or more variables are considered the resultant "p value" was multiplied by all possible comparisons. For example, if 3 groups were used the outcome of A vs. B vs C would be multiplied by 3 (as it would be possible to compare A:B, A:C and B:C) and only if the result was still less than 5% it would be considered statistically significant. In chapter 4, the rate of LTL shortening was estimated as conditional to the baseline measure.

In all studies significance was defined as α-value of less than 5%, indicating that the chance of the null hypothesis still being true even though the difference is greater than the critical value was less than 5%.

3.6 Ethics

All studies presented in this thesis were approved by local research Ethics Committees. As study 4 and 5 involved recruitment of participants in different parts of the UK, multiple local ethics applications were obtained.

4 STUDY 1 - The association between systemic inflammation, oxidative stress and LTL.

4.1 INTRODUCTION

In humans, a common approach used to define the impact of cumulative inflammation on biological pathways involved in disease evolution relies on selection of chronic diseases characterized by high levels of inflammation. For example, diabetes (495;496), PD (497) and rheumatological diseases (498) have been used as models to investigate the possible inflammatory mechanisms involved in the initiation and evolution of CVD. While this approach can be considered reasonable, it does not take into account the variable burden of inflammation which characterizes the different phases of resolution and reactivation of the underlying disease.

Circulating inflammatory markers can inform on acute but not chronic inflammatory burden. For example, CRP is the most common inflammatory indicator used in large epidemiological studies but a statement of the AHA and CDC recommends evaluation of its circulating levels only in metabolically stable patients, underscoring its prognostic limitations in patients with chronic inflammatory diseases (499). In such conditions, CRP may be more affected by fluctuation of the underlying disease activity than providing information on the cumulative inflammatory burden (254-262;497). For example, in patient with PD, the dynamic states of exacerbation and remission, which can be described in terms of patterns of disease progression and regression, are likely to influence circulating levels of inflammatory biomarkers. In this case, a single measure of CRP does not provide information on the cumulative

inflammatory damage related to the oral infection, but will reflect the state of inflammatory activation at the time of blood collection.

Prolonged exposure to high levels of inflammation stimulates adaptive changes which generally occur at the expense of many other physiological processes and cannot be sustained without adverse side effects, including local tissue remodelling and destruction. Therefore, an estimation of the cumulative inflammatory burden can be obtained by measuring the stage of target organ damage. For example, in patients at high CV risk, the inflammatory burden resulting from the lifelong exposure to common CV risk factors can be estimated by measuring the cIMT (marker of vascular remodelling) (500). Similarly, the estimation of the amount of periodontal support around the tooth by probing assessment (clinical attachment levels, CAL) is thought to reflect the cumulative effect of repeated episodes of activation and remission of the underlying inflammatory process (501).

CAL is normally estimated by addition of the probing pocket depth score to the recession score. Probing pocket depth is measured from the free gingival margin to the base of the sulcus or pocket (Figure 4.1). As the free gingival margin can change due to acute inflammatory modifications of the local tissue due, for example, to tissue swelling, probing depth alone do not provide an accurate means to estimate the cumulative gingival damage due to repeated episodes of disease progression and regression. Levels of gingival recession, by contrast, are estimated measuring the distance between cemento-enamel junction and gingival margin (Figure 4.1).

Figure 4.1 Estimation of Clinical Attachment Level (CAL).
Clinical attachment level is obtained from the addition of the probing pocket depth score (distance between the free gingival margin and base of pocket) to the recession score (distance between cemento-enamel junction – CEJ – and free gingival margin). Adapted from web image. Original image available for download at: http://www.newyork-dentalmalpractice.com/glossary/periodontal-disease/.

In case of tissue overgrowth, the gingival margin is coronal to the cemento-enamel junction and the recession score assumes negative values. On the other hand, in case of gingival tissue retraction compared to the cemento-enamel junction the recession score assumes positive values. In this study, CAL was used to inform on the lifetime exposure to the oral infective-inflammatory process**.**

The primary aim of the study was to evaluate LTL in patients affected by periodontitis (exposed to chronic inflammation) compared to controls. The secondary aim was to ascertain the degree of association between measures of chronic inflammatory burden (CAL), Oxidative Stress and LTL.

4.2 Specific methods

4.2.1 Population

In a case control study design, 563 participants were recruited among subjects referred to the UCL Eastman Dental Institute in London between 2002 and 2006. Interviews were used to obtain participant's medical history (including smoking history) and current or previous use of medications. After this preliminary screening, all subjects with medical disorders other than PD (e.g. cancer, type 2 diabetes, hypertension and major CV/endocrine diseases), history and/or presence of other infections, systemic antibiotic treatment in the preceding 3 months, as well as those currently undertaking any medication were excluded from the study.

All recruited patients underwent a clinical periodontal examination performed by a trained periodontist. Full mouth measures of probing pocket depth (PPD), recession (REC; measured as distance from the cemento-enamel junction to the gingival margin), and CAL (measured from the formula CAL=PPD+REC) were obtained at six sites per tooth in all participants. Following oral screening, patients were divided in two groups:

- Cases: individuals diagnosed with severe generalized PD, defined by probing pocket depths greater than 6 mm and marginal alveolar bone loss > 30% with at least 50% of teeth affected (254).
- Controls: subjects without any sites PPD and CAL ≥ 4mm after a basic screening periodontal examination or radiographic evidence of bone loss.

Cases were further divided into those suffering from aggressive or chronic PD according to the American Academy of Periodontology (AAP) 1999 Consensus

classification (289). All patients gave written informed consent and the study was reviewed and approved by the Eastman/UCLH joint ethics committee. Several features make this population unique, such as:

a. Consecutive recruited patients using strict selection criteria which allows excluding possible confounders of the relationship between LTL and inflammatory burden.
b. The large availability of data on a wide range of factors known to influence measures of LTL, allowing extensive adjustments and the independent role of many parameters to be assessed.
c. The oral examination was performed by an experienced periodontist, according to the most recent guidelines.

4.2.2 Biochemical tests

Before the dental examination, blood samples were collected from all patients for biochemical, oxidative stress and LTL assays. Leukocyte counts, lipid profiles, and glucose levels were assessed by standard biochemical testing. A high-sensitivity immunoturbidimetric assay (Tina-Quant CRP assay performed on a Cobas Integra analyser; Roche Diagnostics, inter-assay coefficient of variation = 2.1% on 20 randomly selected samples, lower detection limit 0.03mg/L) was used to measure serum levels of high-sensitivity CRP, while levels of interleukin-6 were measured by a high-sensitivity enzyme-linked immunosorbent assay (Quantikine HS; R&D Systems, lower detection limit 0.038pg/mL). Intra-assay coefficient of variation for the IL-6 measures ranged from 1.9 to 4.9% (each sample in duplicates) while inter-assay coefficient of variation was 4.1% (one sample in duplicate in each plate).

4.2.3 Oxidative stress assays

In this study, D-ROM and BAP tests were used to estimate the total amount of oxidative metabolites and the antioxidant potential of each sample, respectively.

4.2.4 Preparation of the DNA samples and LTL assay

4.2.4.1 DNA Extraction

DNA was already extracted starting from an aliquot of 10-ml EDTA blood sample and using the Nucleons BACC2 kit (Nucleon Bioscience, Coatbridge, UK), as previously described (502). Following extraction, the DNA was diluted in TE buffer (10 mM Tris and 1 mM EDTA) and stored in cryovials at -80°C.

4.2.4.2 DNA Standardization

In each sample the concentration of DNA was standardized following the protocol described in the "general methods".

4.2.4.3 LTL Assay

LTL were measured using the technique described and 14 randomly selected samples were run in duplicates on two consecutive days. There was a significant correlation between the measurements obtained on the two different days using linear regression analysis (R^2= 0.87; P<0.001, Figure 4.2).

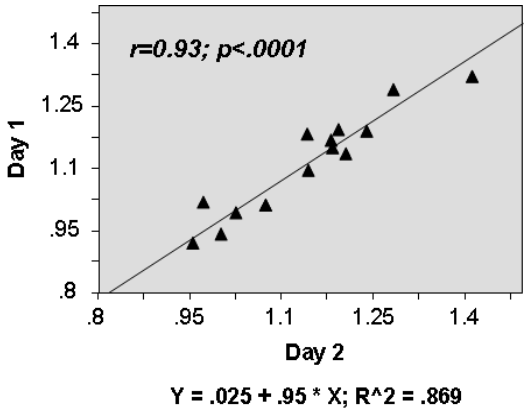


Figure 4.2 Correlation between T/S values obtained at day 1 and day 2 in a subset of the samples.
The strong linear association between day1 and day2 T/S ratios obtained from the same subset of samples confirms the high reproducibility of the LTL assay used in this study.

Moreover, reproducibility was also assessed with Spearman's non-parametric test of pair-wise correlation. The correlation of the lengths' ranking, as measured on the two different days, was highly significant (Spearman Coefficient = 0.97, $P<0.001$). The coefficient of variation of the T/S ratios in the repeated measurements of the same sample was 3.15%, significantly lower than that documented by Cawthon in his original report (5.6%) (82).

4.2.5 Statistical analysis

SPSS (version 17, Windows) was used for all analyses. All data are presented as means and standard deviation (unless differently specified). Age and gender variables were used to identify comparable controls and not to perform individual case-matching. Therefore, they were included as covariates in all analyses. Not-normally distributed variables were log-transformed as appropriate. Differences in continuous variables (including log transformed LTL) between cases and controls were tested with an independent *t* test. From values of T/S ratio, the absolute LTL measure (base pair, bp) was calculated for each sample by means of the regression line resulting from the association between T/S ratio and TRF measures, as previously described. When a statistically significant association was found, multiple linear regression models were fitted, including as covariates age, gender, ethnicity, lipid profile, and smoking differences. Spearman correlation analyses were used to assess the associations between the LTL and the various measures of systemic inflammation, oxidative stress, and PD in both the whole study population and the subgroups (cases and controls). The fully adjusted model was used to compare slopes of *T*/*S* by age in cases and controls. χ^2 analyses were performed to detect differences in categorical variables. The α value for statistical significance for association was set at 0.05.

4.3 Results

356 participants were diagnosed with severe PD (503). The control group was composed of 206 individuals without clinical history or signs of PD or any other disease. A further analysis of the clinical characteristics of the cases identified 71 patients with aggressive PD, while 285 subjects presented clinical features of chronic PD. Clinical and biochemical characteristics of the studied population are reported in Table 4.1, with subjects divided in case and control groups.

Table 4.1 Characteristics of the study population

Variables (mean±se)	**Controls (N=207)**	**Cases (N=356)**	**P value**
Age, years	46.8±0.7	46.9±0.4	0.905
Gender, Male	88(42.5%)	165(47.8%)	0.410
Smoking, Current	86(41.5%)	122(35.4%)	0.291
Ethnicity, Caucasian	126(60.9%)	227(65.8%)	0.463
WBC, cells^6/mm^3	6.1±0.1	6.8±0.1	<0.001
LDL, mmol/l	2.8±0.1	3.2±0.1	<0.001
HDL, mmol/l	1.7±0.03	1.5±0.03	<0.001
Tot Chol, mmol/l	5.0±0.1	5.3±0.1	0.017
Triglycerides, mmol/l	1.3±0.1	1.4±0.1	0.359
CRP, mg/l	1.3±0.5	2.1±0.2	<0.001
IL-6, ng/ml	1.3±0.2	1.2±0.2	0.529
d-ROM, UCarr	277.4±7.8 (N=184)	378.1±10.2 (N=204)	<0.001
BAP, mmol/l	3686.5±140.4 (N=184)	2798.1±121.7 (N=204)	<0.001
T/S ratio	1.22±0.03	1.12±0.02	0.006*

*Values are reported as mean±standard error (se). * P values in fully adjusted models (age, gender, ethnicity, smoking and lipids).*

Individuals with PD had higher levels of inflammatory markers, including CRP (P<0.001) and leukocyte counts (P<0.001) compared to controls, independent of age, gender, smoking and body weight differences. Furthermore, greater total

cholesterol (*P*=0.003) and LDL cholesterol (*P*<0.001) levels, as well as lower HDL cholesterol (*P*<0.001), were observed in cases versus controls. Cases exhibited higher levels of reactive oxygen metabolites (d-ROM, *P*<0.001) and lower antioxidant potential (BAP, *P*<0.001) (Table 4.1).

Mean and standard deviation of LTL in the whole cohort were 1.17 and 0.37 T/S ratio, respectively. As expected, statistically significant inverse correlations were found between LTL and age (R=−0.2, P=0.001) in the whole sample, as well as in individual groups (cases: R=−0.1, P=0.04; controls: R=−0.2, P=0.01). Smokers had significantly shorter LTL compared to non-smokers (1.12±0.31 vs 1.20±0.41 T/S ratio; P<=0.001015). No statistically significant correlations were found between LTL and acute markers of inflammation, such as CRP and IL-6, nor with other biochemical parameters reported in Table 4.1. Notably, cases with PD exhibited shorter LTL compared to the controls (1.23±0.42 vs 1.12±0.31 *T/S* ratio; 8311±2723 vs 7670±2632bp; *P*<0.001). Adjustments for age and smoking as well as for gender and ethnicity (variables that have previously been shown to be associated with LTL in cross-sectional studies) did not materially affect the observed difference between cases and controls (Figure 4.3).

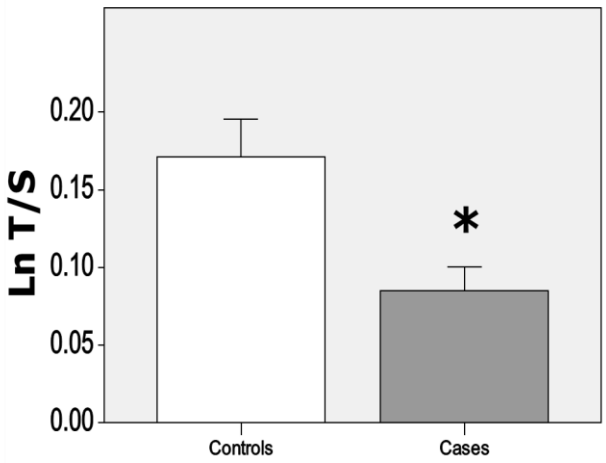


Figure 4.3 Difference of LTL between patient with periodontitis and healthy control
Patients with PD had significantly shorter LTL compared to healthy control (P<0.001). This difference remained significant also after adjustment for age, gender and ethnicity.

In the whole population, as well as in patients with PD, a statistically significant negative association was found between LTL and reactive oxidative metabolites as assessed by d-ROM test (P=0.008, R=−0.2 and R=−0.2, P=0.03, respectively). Similar associations were not found in the control group (R=0.05, P=0.516) (Figure 4.4).

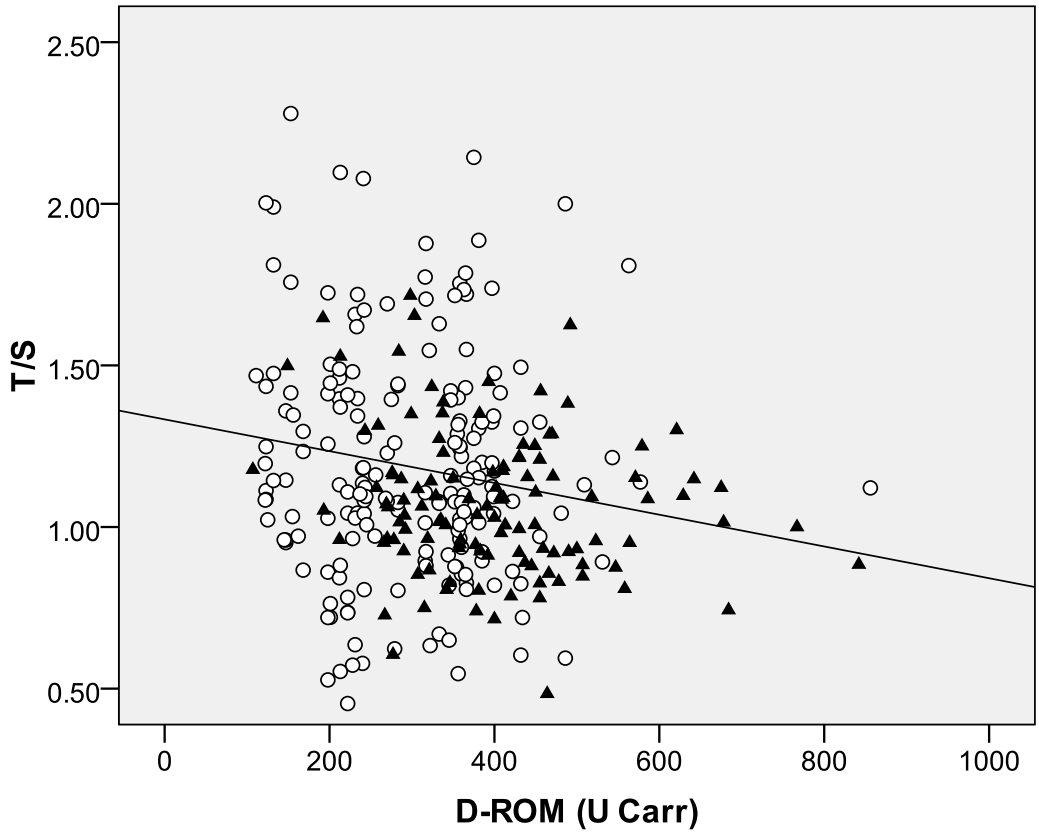


Figure 4.4 Association between LTL and d-ROM.
Scatter plot of leukocyte T/S ratios against serum d-ROM levels (R=-0.2, P=0.001), by Spearman Rank Correlation Test. Controls are drawn as open circles and cases as filled triangles.

Furthermore, CAL was inversely related to LTL (Figure 4.5) and levels of oxidative stress metabolites in cases (P=0.003, R=−0.2 and P<0.001, R=-0.2, respectively) (data not shown).

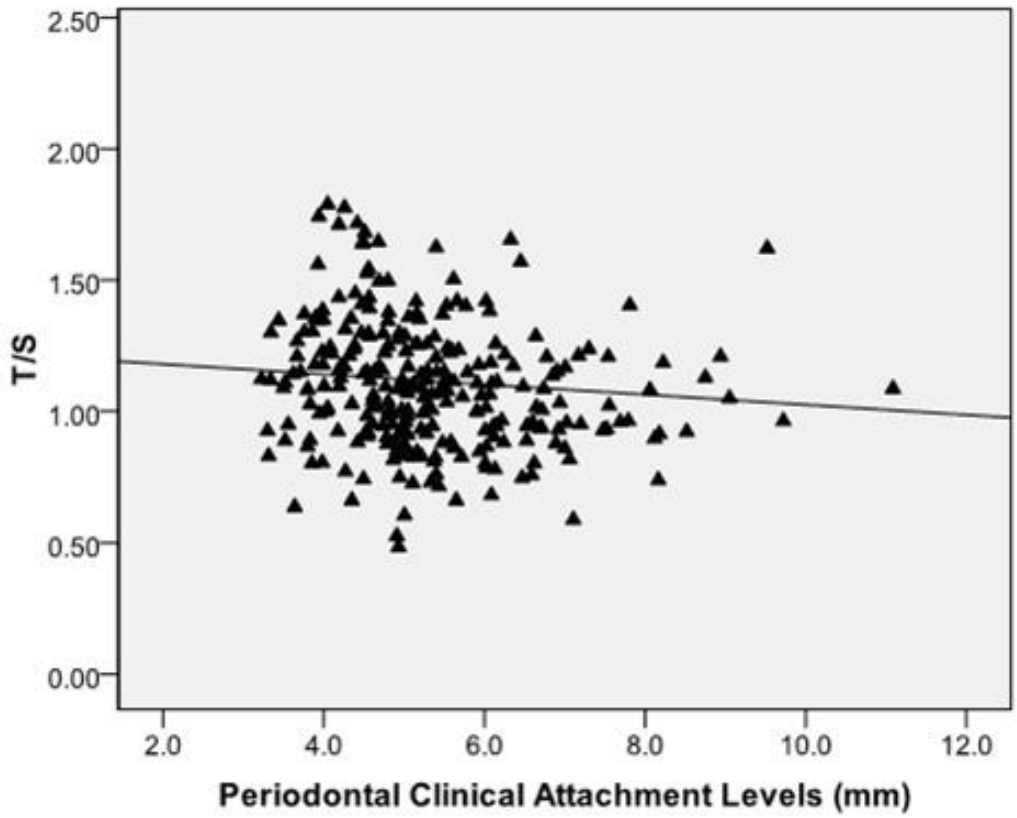


Figure 4.5 Association of CAL with LTL and d-ROM.
There was a statistically significant association between CAL and LTL. This association remained significant after adjustment for age, ethnicity and gender (P=0.003, R=−0.2).

When cases were divided into those with aggressive or chronic periodontal disease, both PD groups exhibited:

a) higher serum levels of CRP ($P<0.01$, Figure 4.6C),

b) higher levels of oxidative metabolites (f-ROM, $P<0.05$, Fig 4.6E),

c) lower antioxidant potential (BAP, $P<0.05$, Figure 4.6F),

when compared to controls.

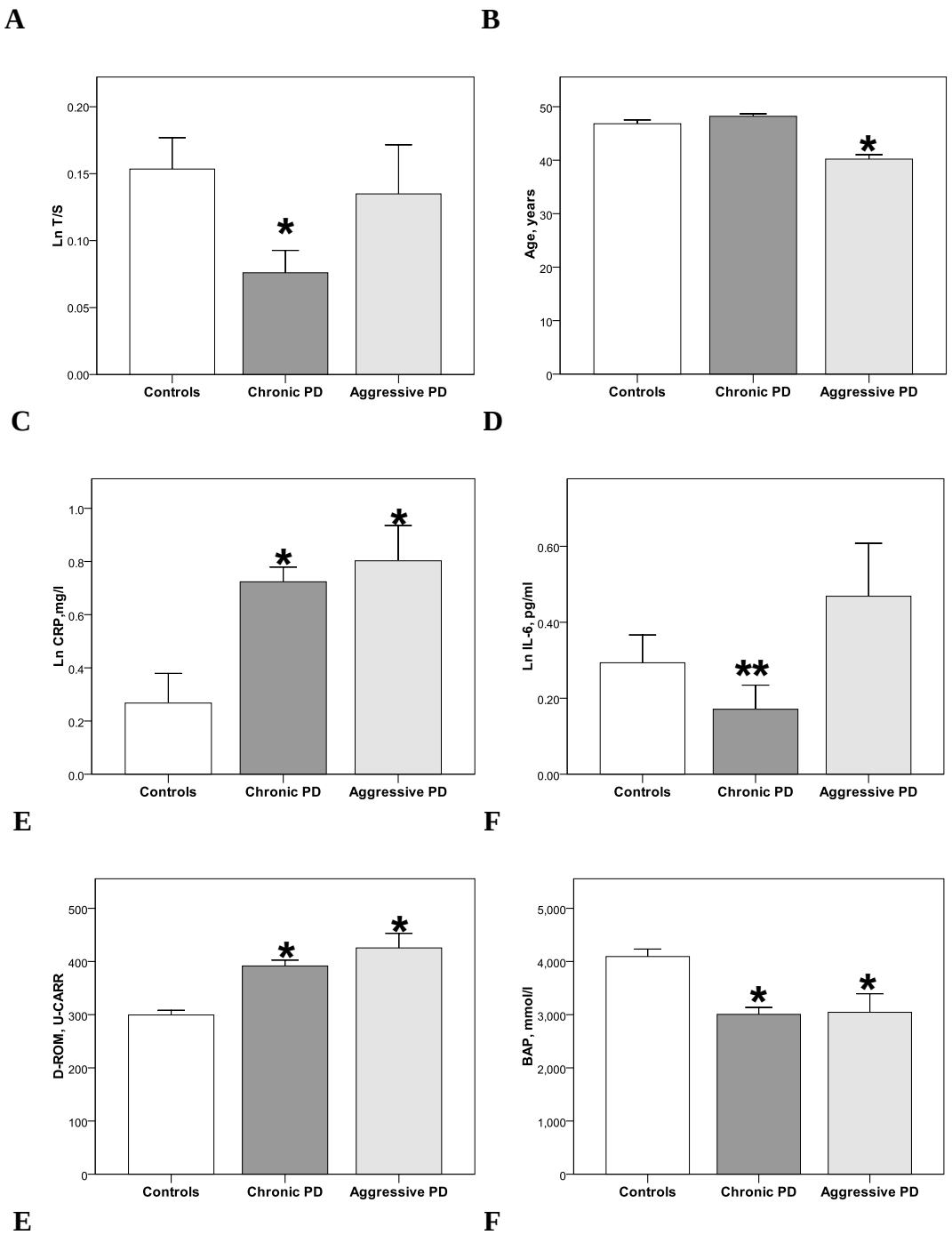


Figure 4.6 Differences of CRP, IL-6, LTL, d-ROM, BAP and Age between aggressive PD, chronic PD and healthy control groups.

Means (± standard errors) of (A) log T/S*, (B) age, (C) log CRP, (D) log IL-6, (E) d-ROM, and (F) BAP of controls (*n *= 207) and chronic (*n *= 285) and aggressive (*n *= 71) periodontitis (PD) cases. *Statistically significant difference (*P *< 0.05) compared to controls using analysis of variance. **Comparison between chronic and aggressive cases using analysis of variance.*

Furthermore, while individuals with aggressive PD were younger (P<0.05, Figure 4.6B) than those with chronic disease and healthy controls, they presented with higher serum IL-6 levels (P=0.048, Figure 4.6D) than chronic cases. LTL was statistically significant shorter in patients with chronic PD compared to healthy subjects (1.12±0.31 *T*/*S* ratio, 7673±2632bp, P=0.01 vs controls), whereas no differences were noted between aggressive cases and controls (1.15±0.33 *T*/*S* ratio, 7807±2632bp, P=0.201), nor between aggressive and chronic cases (P=0.571).

Adjustments for age, ethnicity, gender, and smoking did not materially affect the LTL differences between groups (Figure 4.7).

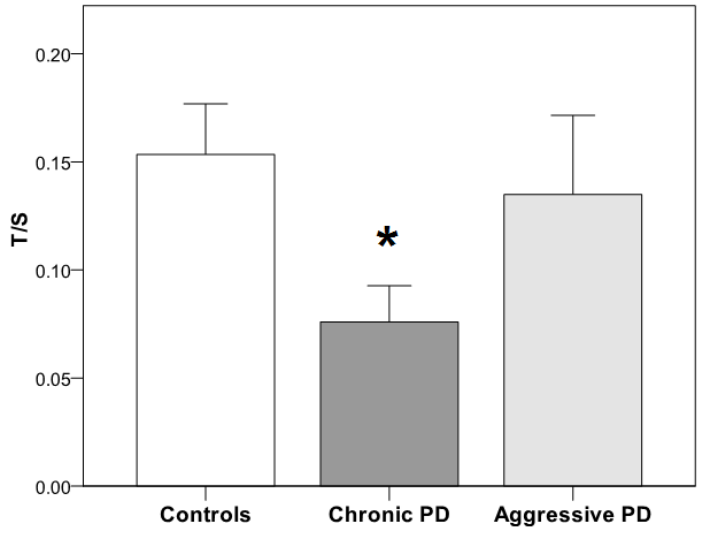


Figure 4.7 LTL differences between aggressive PD, chronic PD and healthy control groups following adjustment for age, ethnicity, gender and smoking.

Figure 4.8 displays the regression plot of the predicted LTL values (obtained from the fully adjusted model which included adjustments for age, gender, ethnicity, and smoking) against the age (in years) of all participants, with regression lines and dots of cases divided from controls.

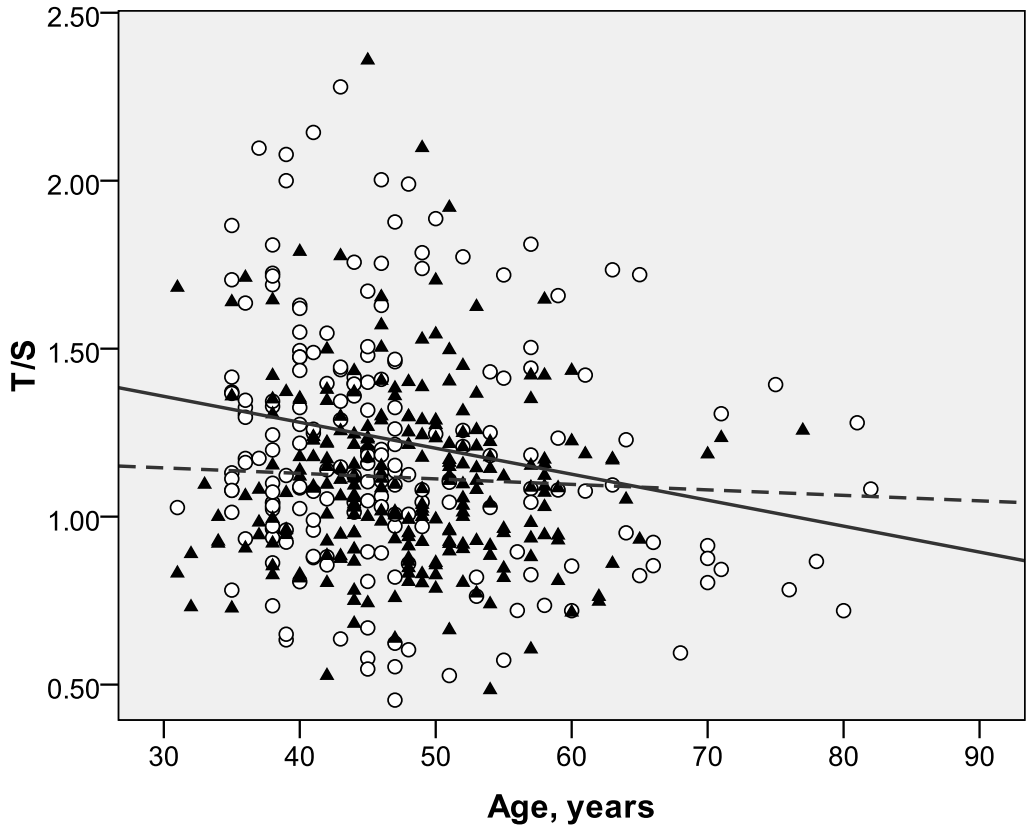


Figure 4.8 Scatter plot of predicted T/S ratios by age in cases and controls.
T/S values are based on fully adjusted models - age, gender, ethnicity, smoking, lipids. Controls are drawn as open circles and cases as filled triangles. Filled line corresponds to the slope of T/S by age in cases and dotted lines to the slope of T/S by age in controls. P value of statistical difference in slope between cases (slope=0.002) and controls (slope=0.035) is 0.001.

A steeper age slope was observed in cases (P=0.01) versus controls, suggesting a faster rate of LTL shortening in the population of PD patients compared to healthy subjects.

4.4 Discussion

This is the first report reporting an association between shorter LTL and PD. Diagnosis of PD was associated with shorter LTL, higher systemic inflammation and oxidative stress exposure compared to controls. Importantly, LTL was negatively correlated with total levels of reactive oxidative metabolites detected by d-ROM test and with severity of PD, assessed by clinical attachment level. These associations were independent of age, gender, ethnicity, and smoking differences. In subgroup analyses, LTL was shorter only in patients with chronic disease compared to controls. A diagnosis of aggressive PD was not related with shorter LTL, although levels of systemic inflammation in these patients were higher than those recorded in subjects with chronic disease.

Takahashi et al previously analysed the association between PD and LTL. They reported no differences in LTL between subjects with and without oral disease (504). Their study, however, has important limitations. Firstly, the small number of patients might have precluded the identification of real differences in LTL as there is high inter-individual variability of LTL. Secondly, they enrolled only patients with aggressive disease, who are unlikely to have shorter LTL compared to healthy controls, as confirmed by our results.

According to previous reports, PD patients showed increased levels of systemic inflammatory markers and oxidative metabolites (295;505;506), while their biological antioxidant potential was reduced compared to the control group. As inflammation and oxidative stress strongly affect the rate of LTL shortening (33;42;62), it is possible that the shortened LTL in PD patients resulted from long term exposure to these environmental factors. The chronic inflammatory response in patients with PD

induces an increase in the numbers of leukocytes in the circulation. Furthermore, the continuous bacterial aggression to the periodontium causes a heightened expenditure of inflammatory cells in the local inflammatory process, as they are sequestered and die in the attempt to remove the inflammatory trigger. The demands to maintain the numbers of leukocytes would promote an increase replication of cells up the hierarchy of the hematopoietic system, which ultimately involve the HSCs. The higher rate of replication and differentiation of HSCs determine a faster rate of telomere attrition due to the "end-replication problem". This process is only partially compensated by telomerase, as several studies have demonstrated that, also in HSCs with residual enzyme activity, it is possible to detect progressive telomere attrition with subsequent cell divisions (507;508). Thus, the faster rate of telomere attrition in HSCs will be reflected in shorter telomeres of their daughter cells, the peripheral leukocytes (113).

A further contribution to shortened LTL in patients with periodontitis might be provided by the increased levels of oxidative stress. Indeed, higher levels of oxidative stress augment the number of telomere repeats being lost per cell replication by direct or indirect mechanisms (33;42). The direct damage is dependent on double or single strand break of the telomeric DNA during cell division, which cannot be adequately compensated or repaired by telomerase activity (47). Furthermore, hydroxyl radicals can cross-link with guanine bases, forming oxidised products (8-oxodG) which stop elongation of telomeric repeats by telomerase and lead to a variable number of telomeric repetitions which remain un-replicated during each cell division (48;125). Over and above the impact on HSCs, these mechanisms of oxidative stress-mediatedtelomere damage are likely to be particularly relevant in subsets of leukocytes which replicate in the peripheral circulation, such as lymphocytes. As

DNA polymerase is less efficient in repairing the telomeric than the genomic DNA following oxidative stress damage (47), the size of telomeres has been suggested as a measure of an individual's cumulative burden of oxidative stress (509). Our study confirms this potential role for telomeres, as LTL not only related with circulating levels of reactive oxidative species but also with markers of chronic evolution of PD, such as clinical attachment levels. Indeed, it is now well established that an excess of reactive oxidative metabolites, coupled with a depletion of the antioxidant capacity in gingival crevicular fluid (287;510;511), might be responsible for the chronic local activation of periodontal inflammation and progressively lead to gingival tissue destruction. Therefore, increased levels of oxidative stress might represent the final mediators of the association between LTL and clinical attachment levels.

In contrast with this hypothesis was the finding that markers of systemic inflammation did not correlate with LTL, and patients with aggressive PD had higher levels of IL-6 but did not show shorter LTL. Several explanations might account for these findings. Firstly, fluctuations in the burden of bacterial aggression can cause acute changes of systemic inflammatory markers in patients with PD, making levels of CRP and IL-6 not informative on the cumulative exposure to inflammation (255;292). LTL dynamics are unlikely to be affected by acute changes of the inflammatory response. This might explain why LTL was not associated with inflammatory markers as well as why CAL, a clinical marker related to duration of PD, was associated with LTL but not with CRP or IL-6. Secondly, in our study, patients with aggressive PD were younger than those with chronic PD. This was an expected finding, as the clinical manifestations of aggressive PD tend to appear earlier and with worse presentations than those in chronic disease (274;289). Consequently,

while it is not surprising that inflammation and oxidative stress exposure were higher in aggressive patients than in the other two groups, these environmental factors could not have enough time to impact significantly on the slow LTL dynamics.

The comparison between T/S ratio of our Q-PCR assay and TRF analysis had been previously reported (479). By means of the regression line resulting from this correlation, we calculated the corresponding telomere length in bp from the T/S ratio measured in each subject. These calculations resulted in a difference in LTL between cases and controls of 641 bp, reflecting a biological aging gap of approximately 21 years (112). Although the role of LTL as marker of biological age is not completely accepted, there is evidence that patients with PD experience a higher mortality compared to healthy subjects (512;513). Söder et al demonstrated in a large longitudinal prospective study that young adults with PD have a significantly higher risk or premature death from neoplasms and CVD (512). These diseases are typically related with shorter LTL (61;68;69;352-354) and LTL has been shown to be inversely related with mortality rate (58;69;514). Thus, our current results provide a possible molecular pathway explaining the previously reported epidemiological associations.

The observational experimental design of this study cannot exclude reverse causality (i.e. that shorter LTL could increase the risk of PD or accelerate the evolution of the disease). LTL is a marker of cellular aging and cells with critically short telomeres undergo apoptosis or senescence (28;40). As cells developing such a phenotype can no longer replicate, their accumulation in different tissues and organs marks a reduced ability to repair and leads to an increased susceptibility to environmental damage. Therefore, the shortened telomeres detected in patients with periodontal clinical attachment loss could result from deficient replication and repair

capacities of the local gingival fibroblast. Although we did not measure gingival fibroblast telomere length, a large body of evidence has shown that there is close correlation of telomere length in different tissues from the same individual (88-90;113;515;516). It is therefore possible that people with short telomeres in their peripheral leukocytes also have short telomere length in their gingival tissues.

Telomere length not only acts as an internal mitotic clock for somatic cells but also plays a key role in regulating several cellular functions, including the ability to produce pro-inflammatory cytokines (242). Therefore, the shortened LTL observed in PD patients could mark a general dys-regulation of the immune and inflammatory responses, which has been long considered as a possible reason for the higher susceptibility to the PD itself. As a result, the shortened LTL of PD patients may have a central role in perpetuating the oral inflammatory disease. The idea of a causal role for LTL in the initiation and evolution of PD, is supported by evidence that individuals with some genetic diseases associated with accelerated telomere loss in peripheral leukocytes, such as Down's syndrome, show an increased risk of PD when compared to controls (517). Further research is needed in this area, possibly including studies with longitudinal LTL measurements in patients with PD.

A number of limitations should be discussed when interpreting our results. Firstly, both d-ROM and BAP are global measures of RS and therefore cannot inform on which exact RS or antioxidants have the stronger relation with PD and short LTL. Moreover, the lack of data on telomere length from gingival fibroblasts do not enable testing of whether, the loss of telomere length in peripheral leukocytes reflects a concomitant and more generalized aging process affecting in addition gingival tissues. While Takahashi et al did not find a significant correlation between LTL and gingival

fibroblast telomere lengths, this relationship was explored in only a few patients with the aggressive disease and further research is needed (504). Finally, the quantitative PCR-based assay measures the average telomere length across all leukocytes in the peripheral blood. Because of the significant difference in the total leukocyte count between cases and controls, we cannot exclude the possibility that the chronic inflammatory state typical of patients with PD might have shifted the composition of leukocyte subpopulations in a way that favoured cells with shorter telomeres. Despite this, shorter telomeres in a specific leukocyte subpopulation predict the presence of short telomeres in other subgroups of mature white cells and hematopoietic progenitor cells (113;515). This suggests that our results are more likely to represent a generalized aging process involving all the different white cells in the peripheral blood as an expression of their common origin from the bone marrow hematopoietic stem cells.

In conclusion, our data suggest that a chronic exposure to inflammation could represent the main cause of the short LTL recorded in patients with PD, with circulating levels of oxidative stress acting as the main mediator of the association between inflammatory burden and short LTL. Moreover, the evidence of shorter LTL detected in individuals with chronic periodontitis could provide a possible biological explanation for the reported higher mortality rate in these patients compared to the general population. Further research and longitudinal studies, however, are needed to confirm the prognostic importance of these findings.

KEY POINTS

Chronic inflammation is likely to have an impact on LTL as suggested by the association between markers of chronic inflammation and LTL

Circulating levels of inflammatory markers are unable to capture the relationship between chronic inflammation and LTL

Oxidative stress exposure is likely to represent the mediator of the relationship between chronic inflammation and short LTL

LTL could represent a biological pathway explaining the higher mortality risk observed in patients with PD

5 STUDY 2 - The association between LTL and markers of chronic inflammation is independent from the short term metabolic control

5.1 Introduction

The second study explored whether, the association between short LTL and chronic inflammatory burden is independent from metabolic factors or concomitant diseases. A cross-sectional sample of patients with type 1 and type 2 diabetes mellitus was screened for presence of moderate to severe periodontal pockets versus gingivitis and LTL were measured. Circulating levels of LPS (which could reflect the amount of bacterial load on the periodontal tissue) and metabolic parameters were correlated with LTL, suggesting that continuous activation of the immune-inflammatory response may be a possible cause of LTL attrition. Finally, the study investigated whether a higher inflammatory burden reflected by the presence of shorter LTL was associated with diabetic complications (i.e. diabetic nephropathy).

5.1.1 Diabetes and LTL

The first report showing shorter LTL in patients with type 1 diabetes was published by Jeanclos et al and consisted of a cross-sectional, case-control study including 234 white men, of whom 54 had type 1 diabetes, 74 had type 2 diabetes, and 106 were control subjects (518). LTL was shorter in patients with type 1 but not in those with type 2 diabetes, compared to the control group. Subsequently, several studies have analysed the cross-sectional association between LTL and diabetes, reporting inconsistent results. In the Framingham Heart Study, Aviv et al. found shorter LTL in subjects with insulin resistance (62). However, the same group could not replicate these findings in the population of the Cardiovascular Health Study (63). In a longitudinal analysis involving a small group (n=49 participants) of the Bogalusa

Study, Gardner et al reported that the relative changes in telomere length over 10.1 to 12.8 years were correlated with changes in insulin resistance and body mass index (66). In contrast, in a much larger recent study no associations were reported between diagnosis of type 2 diabetes and LTL, and LTL at baseline did not predict development of diabetes in 6 years of follow-up (519).

The association between LTL and diabetes has usually been explained by the high levels of oxidative stress and inflammation normally found in patients with diabetes. Sampson et al showed increased oxidative DNA damage and shorter LTL in all subsets of circulating monocytes of diabetic patients compared to healthy controls (76). Importantly, a significant inverse relationship was described between levels of oxidative DNA damage and telomere length in the diabetic group. Similar results were obtained by Salpea et al, who studied a large population of 742 type 2 diabetes patients and 448 healthy controls (250). Diabetes patients had shorter LTL compared to healthy controls and plasma total antioxidant status was positively associated with the measure of LTL only in the diabetes group. Moreover, diabetes carriers of the functional variant (-866G>A) in the promoter of human *UCP2* gene (associated with increased exposure to oxidative stress of mitochondrial origin) had shorter LTL than the more common homozygotes (-866G>G).

The ability of LTL to mark chronic inflammation and oxidative stress exposure in diabetes may explain the higher rate of complications in patients with shorter LTL. Olivieri et al reported that type 2 diabetes patients with history of myocardial infraction have shorter LTL than those without CV complications (520). More recently, the same group demonstrated that among patients with diabetes, LTL became significantly and gradually shorter with the increasing number of diabetes complications (521).

However, the variable association reported between shorter LTL and diagnosis of diabetes suggests that, over and above the degree of inflammatory burden due to the metabolic disease, other factors may affect LTL dynamics. Diabetes care is complex and requires a multidisciplinary approach as many issues and comorbidities may influence levels of inflammation, the quality of metabolic control, the risk of complications and, eventually, patient outcomes (522). One of the most common comorbidities observed in diabetes patients is PD. Epidemiological data suggests susceptibility to PD is increased by approximately threefold in people with poorly controlled diabetes (523). Additionally, the risk of cardiorenal mortality and end-stage renal disease is three times higher in the diabetes population with severe PD than those without oral disease (301). However, biological pathways involved in increasing the risk of complications in patients with diabetes and PD remain unclear.

Our first study suggested that the chronic inflammatory burden reflected by severity of periodontal remodelling/destruction may represent the main driver of shorter LTL in patients with PD. In the second project of this thesis, the association between severity of periodontal inflammation and short LTL was assessed with regards to metabolic factors, representing a possible biological pathway underlying the evolution of diabetes complications (i.e. diabetic nephropathy).

A population of type 1 and type 2 patients with diabetes was screened for prevalence periodontal inflammation, in order to verify whether, the severity of gingival inflammation was related to shorter LTL, independently of the gluco-metabolic control. Further, the influence of this association on the prevalence of diabetic nephropathy was assessed.

5.2 Specific Methods

5.2.1 Population

A total of 630 consecutive individuals referred to the Endocrinology Department of the University College of London Hospital were recruited for this study between May 2007 and September 2009 if willing to receive a periodontal screening and blood sample (371 with type 2 and 259 with type 1 diabetes mellitus based on the recent WHO definition). A physician collected participants' clinical (diagnosis, disease duration, complications) and smoking history (current, former or never). Medical records were screened by a separate investigator to obtain information on current medications. Anthropometric measurements and blood pressure were assessed using standard techniques. After consenting, all participants underwent blood collection first and then an oral examination performed by two expert periodontists (NG, FD). Ethical approval was received from the NRES Committee London – Bentham (06/Q0502/97) and written informed consent was obtained from all participants.

5.2.2 Periodontal examination

Measures of exposure to periodontitis are a controversial matter for debate and our group has previously supported the use of both continuous and categorical measures of periodontal exposure when available in epidemiological studies (D'Aiuto et al. J Clin Endocrinol Metab. 2008 Oct;93(10):3989-94; Stewart et al. Psychosom Med. 2008 Oct;70(8):936-41; Tsakos et al. J Hypertens. 2010 Dec;28(12):2386-93; Suvan et al. Obes Rev. 2011 May;12(5):e381-404; Tu et al. J Clin Periodontol. 2013 Aug 14). In this survey the periodontal examination consisted of a single recording of periodontal probing pocket depth based on the Basic Periodontal Examination (BPE)

index derived from the WHO CPITN score (524;525). This screening tool was performed in each sextant of the whole dentition (scores ranging from 0 to 4) on and the highest score of the whole mouth was entered as representative of the periodontal bleeding and deeper probing pocket depth status for each participant. Radiographic assessment of current bone levels and more detailed whole mouth gingival assessments could not be obtained. According with the BPE score, patients were grouped as follows:

- Group 1 – corresponding to a BPE score of 1 and 2, These included all patients without PPD >3.5 mm but either bleeding after probing without calculus/overhangs (Score 1), or bleeding after probing and carrying supra- or subgingival calculus/overhangs (score 2).
- Group 2 – corresponding to BPE score 3 which indicate PPD of 4 to 5mm.
- Group 3 – corresponding to BPE score 4 which indicates PPD of 6mm or more.
- Group 4 – corresponding to patients with no teeth (edentulous).

No individuals presented with BPE scores 0 which indicates no pockets >3.5 mm, no calculus/overhangs and no bleeding after probing. In addition, the sum of all sextants (BPE cumulative score) was created to define a continuous measure of extent of the disease. Both examiners were previously calibrated on a convenient sample of 10 individuals and k scores were calculated (>0.90).

Every participant with BPE scores greater than 2 was asked to attend a further visit for a full periodontal examination (consisting of 6 point full mouth periodontal probing pocket depth, gingival recession, clinical attachment levels and gingival bleeding upon probing by a single calibrated examiner).

5.2.3 Biochemical tests

Blood samples were collected from all patients after an overnight fast for biochemical tests and telomere length assay. Lipid profile, glucose, glycated haemoglobin (HbA1c), creatinine and CRP levels were assessed on an automated analyser (Cobas Integra analyser; Roche Diagnostics). Estimated glomerular filtration rate (eGFR) was calculated with the abbreviated MDRD (Modification of Diet in Renal Disease Study) equation and expressed in mL/min/1.73m^2 (526).

Serum levels of insulin were quantified with high sensitivity ELISA (Mercodia, Sweden), which uses a direct sandwich technique (described in the general methods) and does not cross-react to C-peptide or proinsulin. Insulin resistance index was calculated as previously described (527). Serum endotoxin activity was determined by the Limulus Amebosyte Lysate test kit with a chromogenic substrate (Lonza, Walkersville, MD, US). The steps of the LPS assay are described in the general methods. Intra- and inter- coefficients of variation for all assays were < 5%.

5.2.4 DNA preparation, standardization and LTL assay

5.2.4.1 DNA preparation, standardization

DNA was isolated, quantified and standardized from an aliquot of EDTA whole blood stored at -80°C, using the protocols described in the "general methods".

5.2.4.2 LTL assay

The real time PCR assay described in the general methods was used to measure LTL in a blinded fashion. To test the reproducibility of the qPCR technique in this cohort, a subset of 16 randomly selected DNA samples were run on two different days. The r^2 of the correlation between the 2 runs was 0.92.

5.2.5 Statistics

All data is presented as mean and standard deviations unless otherwise specified. All biochemical variables were log-transformed if normality assumptions were not met. Descriptive analyses were performed on all survey participants, comparing a number of confounders/variables across different levels of periodontal exposure (Groups 1-4) using a test for trend (Jonckheere Trend Test) for continuous and chi-square test for categorical variables. Non-parametric correlation analyses between LTL and age, body composition, and biomarkers were performed using Spearman Rank testing. Linear regression models were used to investigate the univariate association between LTL and all common confounders. All those factors found to be statistically significant at the 0.10 level in the univariate analyses, were then included in generalized linear models. Adjustment was made for: Age, Gender, Ethnicity, Diabetes Type, Smoking, Waist circumference (Model 1) and in addition for Endotoxin, Medication use, Insulin and CRP (Model 2). Fully adjusted models were used to compare slopes of LTL by age across groups of prevalent periodontal inflammation. Despite the limited sample size of the group of edentulous individuals, all analyses were performed including and excluding this group in all multivariate models in order to confirm the results (data are presented with this group included).

229 (70%) of the 327 participants of the survey who had deeper periodontal pockets (Group 2-3) received a full mouth periodontal examination. In this subset sample we used the most current case definition of periodontitis (based on the CDC and AAP consensus, Eke et al J Periodontol. 2012 Dec;83(12):1449-54) and performed a ROC discriminatory analysis to define the sensitivity and specificity of this definition with our categorization of Group 2 and Group 3 as moderate and severe periodontitis. The analysis confirmed high sensitivity (0.85) and low specificity (0.4) scores when using

BPE as measure of exposure for a clinical diagnosis of periodontitis. Further we cross-tabulated Groups 2 and 3 allocations against the CDC-AAP diagnoses of moderate and severe periodontitis respectively. When using BPE scoring the analysis underestimated the diagnosis of severe versus moderate periodontitis in 25% of cases. Further the BPE cumulative score was positively correlated with common periodontal clinical parameters of severity and extent: the number of periodontal pockets greater than 4mm ($R=0.6$, $p<0.001$), full mouth probing pocket depths ($R=0.4$, $p<0.001$) and clinical attachment levels ($R=0.2$, $p=0.023$).

5.3 Results

A total of 255 participants were included in Group 1 with only gingival bleeding (sign of gingivitis), 327 with deeper periodontal pockets (114 with moderate and with 213 severe pockets) and 48 individuals were edentulous (Table 5.1).

Table 5.1 Demographic, anthropometric and biochemical characteristics of the population.

Variables (mean±sd)	**Group 1 (N=255)**	**Group 2 (N=114)**	**Group 3 (N=213)**	**Group 4 (N=48)**	**p for trend**
Age, years	50.0±17.3	58.3±15.4	61.7±11.5	71.6±8.2	<0.001
Gender, Male	131(51.4%)	79(69.3%)	138(65.1%)	26(54.2%)	0.002
Smoking, Current	33(12.9%)	26(22.8%)	30(14.1%)	7(15.2%)	0.022
Ethnicity, Caucasian	203(79.6%)	81(71.7%)	119(55.9%)	29(60.4%)	<0.001
Diabetes, Type II	104(40.8%)	69(60.5%)	154(72.3%)	44(91.7%)	<0.001
BMI, kg/m^2	27.5±6.6	28.8±7.0	28.7±9.2	30.0±5.1	0.004
Waist circumference, cm	100.1±13.1	103.4±12.1	103.1±16.1	108.1±11.0	<0.001
Systolic BP, mmHg	129.0±19.7	131.4±21.0	131.2±18.2	136.1±16.7	0.029
Diastolic BP, mmHg	74.4±11.3	77.2±11.0	75.2±12.0	74.4±11.5	0.570
HbA1c, %	8.0±1.6	7.9±1.5	8.1±1.7	7.9±1.8	0.811
Glucose, mmol/l	8.4±4.8	8.0±4.9	8.5±4.9	8.6±4.0	0.834
Insulin, pmol/L	79.5±115.5	120.8±187.9	118.2±148.3	212.6±262.8	<0.001
HOMA Index	0.1±7.4	1.1±11.9	1.3±12.5	2.1±4.5	<0.001
LPS, EU/ml	10.9±8.0	10.4±10.6	9.8±7.0	12.2±10.0	0.04
CRP, mg/l	1.3±4.0	1.6±7.3	1.5±5.3	2.9±8.5	0.002
LDL, mmol/l	2.3±0.8	2.3±0.9	2.1±0.9	2.0±0.9	0.001
HDL, mmol/l	1.3±0.5	1.1±0.5	1.1±0.4	0.9±0.3	<0.001
Tot Chol, mmol/l	4.4±0.9	4.3±1.1	4.1±1.0	4.0±1.0	0.002
Triglycerides, mmol/l	1.1±0.8	1.2±1.1	1.2±.08	1.5±0.9	<0.001
Creatinine,mg/l	78.5±36.9	80.5±30.1	82.0±56.4	105.0±104.3	<0.001
eGFR, $ml/min/1.73m^2$	86.6±27.9	86.3±29.2	82.3±27.0	57.3±30.8	<0.001

p values are calculated from test for trend, categorical variables compared with χ^2 test

All groups with increased signs of periodontal inflammation were generally older, with higher prevalence of males, more Caucasians and type 2 diabetes diagnosis, but lower number of current smokers, when compared with the Group 1. A linear trend of greater values of systolic and diastolic blood pressure as well as adiposity (both body mass index and waist circumference) was observed in individuals with more severe gingival inflammation and the difference was greatest in the edentulous group. There were no statistically significant differences in HbA1c, plasma glucose levels between groups, whilst levels of insulin, insulin resistance (calculated based on the HOMA IR index), CRP, Endotoxin, triglycerides and creatinine were higher in individuals with increasing BPE scores. An inverse relationship was observed between serum levels of CRP and BPE scores based on diabetes diagnosis. Indeed, whilst in patients with type 1 diabetes Groups 3 and 4 were associated with lower levels of inflammation, the opposite was observed in type 2 patients (CRP levels in Groups 3 and 4 were higher than those in Groups 1 and $2p<0.01$). No association was found between LTL and CRP levels (data not shown).

All study participants with deeper PPD (>4mm, BPE score 3 and 4, corresponding to groups 2 and 3) had shorter LTL when compared with healthier gingival scores ($p=0.04$ for the comparison of groups 2+3 vs group 1, when adjusted for age), and this difference remained statistically significant after multiple adjustments (Figure 5.1).

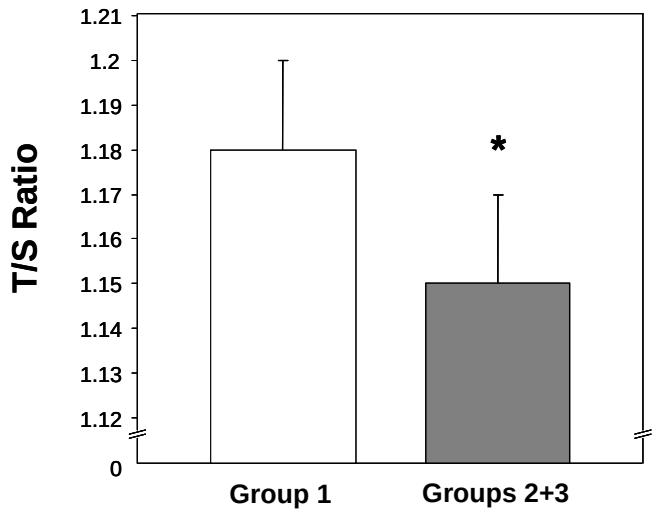


Figure 5.1 Difference in LTL between group 1(participants with BPE scores of 1 and 2, n=280) and groups 2+3 (participants with BPE scores of 3 and 4, n=327) in a cohort of individuals with diabetes.
*Cases with deeper periodontal pockets had shorter LTL compared to those in Group 1. Data are reported as mean ± SE and analysis is adjusted for age, gender, ethnicity, diabetes type, smoking, waist circumference, endotoxin, insulin and CRP levels. *<0.05 compared to gingivitis.*

When results were analysed by increasing BPE scores, cases with higher scores (deeper periodontal pockets) exhibited shorter LTL when compared to group 1, independently of other confounders (Table 5.2).

Table 5.2 Means (± SE) telomere length (T/S ratio) of 630 individuals with diabetes mellitus by severity of BPE score.

	Group 1 (N=255)	Group 2 (N=114)	Group 3 (N=213)	Group 4 (N=48)	p value†
Age-adjusted	1.13±0.01	1.13±0.02	1.10±0.01*	1.19±0.03	0.04
Model 1	1.16±0.02	1.17±0.03	1.12±0.02*	1.22±0.04	0.01
Model 2	1.16±0.02	1.17±0.03	1.12±0.02*	1.23±0.04	0.002

† p value for difference in BPE scores Groups unordered
Model 1: adjusted for Age, Gender, Ethnicity, Diabetes Type, Smoking, and Waist circumference.
Model 2: Model 1 + circulating levels of Endotoxin, insulin and C-Reactive protein.
** P<0.05 compared to Gingivitis*

Edentulous individuals, despite being older, did not have shorter LTL compared to Group 1 in the fully adjusted model. The BPE cumulative score (beta= -0.003, 95%CI -0.005 to -0.001, p=0.013) and circulating levels of endotoxins (beta= -0.027, 95%CI -0.052 to -0.027, p=0.034) were independently associated with LTL when adjusting for all confounders. Indeed there was a negative linear association between LTL and increased BPE scores (Figure 5.2A) and circulating endotoxin levels (Figure 5.2B).

A **B**

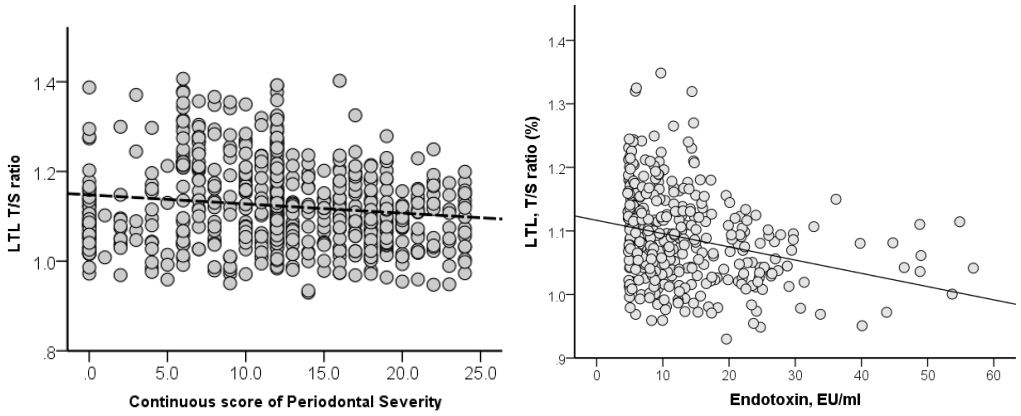


Figure 5.2 Association between LTL (T/S ratio) and cumulative BPE score in each individual and circulating level of LPS.
There was an negative association between LTL and BPE scores (A)(R= -0.11; P=0.009), as well as between LTL and LPS levels (B) (R= -0.29; P<0.001). Analysis adjusted for age, gender, ethnicity, diabetes type, smoking, waist circumference, and circulating levels of LPS, insulin and C-Reactive protein.

When the association between LTL and endotoxin levels was analysed by subgroup of BPE scores, a stronger negative correlation was found in patients with deeper (>4mm, group 3 and 4, r= -0.14; p=0.04) than in those with mild (<3.5mm, group 1 and 2) PPD (Figure 5.3).

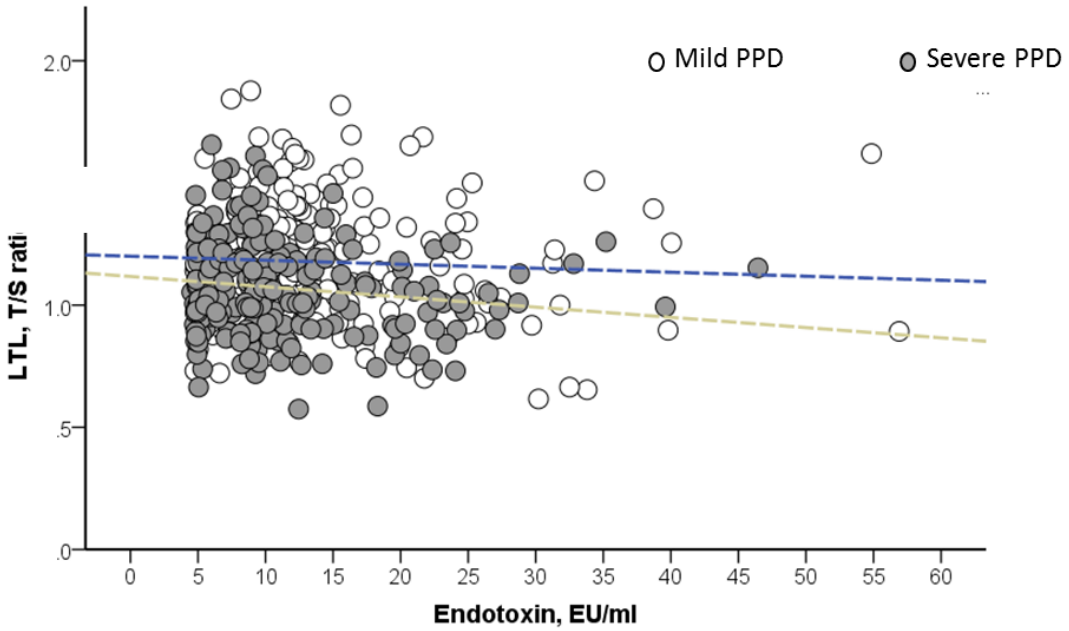


Figure 5.3 Scatter plot of predicted T/S ratios vs endotoxin values in individuals with mild and severe PPD based on BPE scores.
Endotoxin values are log-transformed. Analysis was adjusted for age, gender, ethnicity, smoking, waist circumference and diabetes type. P value of statistical difference in slope between mild (slope=0.023) and severe PPD (slope=-0.051) is 0.001.

Patients with deeper periodontal pockets as well as edentulous presented with higher levels of endotoxemia when compared to cases with mild PPD in the group of type 2 diabetes (13.2±1.6 EU/ml severe PPD and 14.5±1.9 EU/ml edentulous versus 10.5±1.2 EU/ml in the gingivitis group, p=0.025 and p=0.026 respectively). An association of opposite direction (lower endotoxin levels with higher level of periodontal inflammation) was observed in type 1 diabetes (p<0.001) (data not shown).

Indeed, combined Groups 2 and 3 had shorter LTL than Group 1 in individuals with type 2 (p=0.039) but not type 1 diabetes (Figure 3). A progressive reduction of LTL was noted with increasing severity of BPE scores only in cases with type 2 diabetes. Those individuals with prevalent deeper periodontal pockets (Group 3)

showed shorter LTL when compared to the individuals with mild PPD or no PPD (Group 1)(p=0.004) (Figure 5.4).

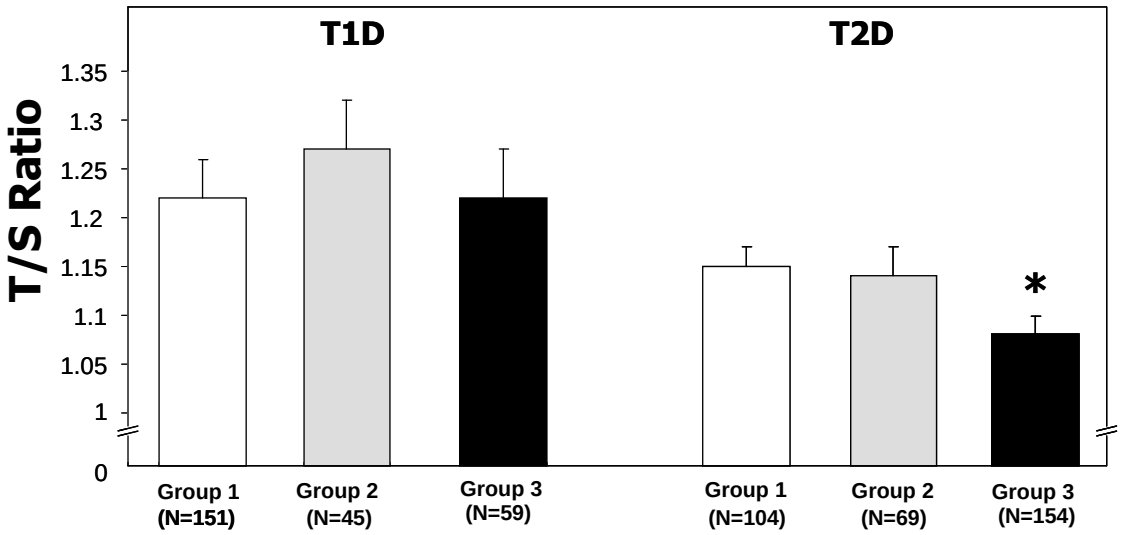


Figure 5.4 LTL (T/S ratio) of 630 individuals sorted by diabetes type and severity of BPE scores.

*Data are reported as mean ± SE and analysis is adjusted (according to Model 2) for age, gender, ethnicity, diabetes type, smoking, waist circumference and circulating levels of Endotoxin, insulin and C reactive protein. *P<0.01 vs group 1.*

Amongst all metabolic parameters a strong association was only found between LTL and insulin resistance (HOMA IR index) (beta= 0.001, 95%CI 0.001 to 0.002, p=0.002) in the fully adjusted model.

Lastly, LTL and eGFR were linearly associated (r=0.164; p<0.001) and adjustment for diabetes type, ethnicity, smoking, waist circumference and blood pressure did not affect the association (beta= 0.001, 95%CI 0.001 to 0.002, p=0.002) (Figure 5.5).

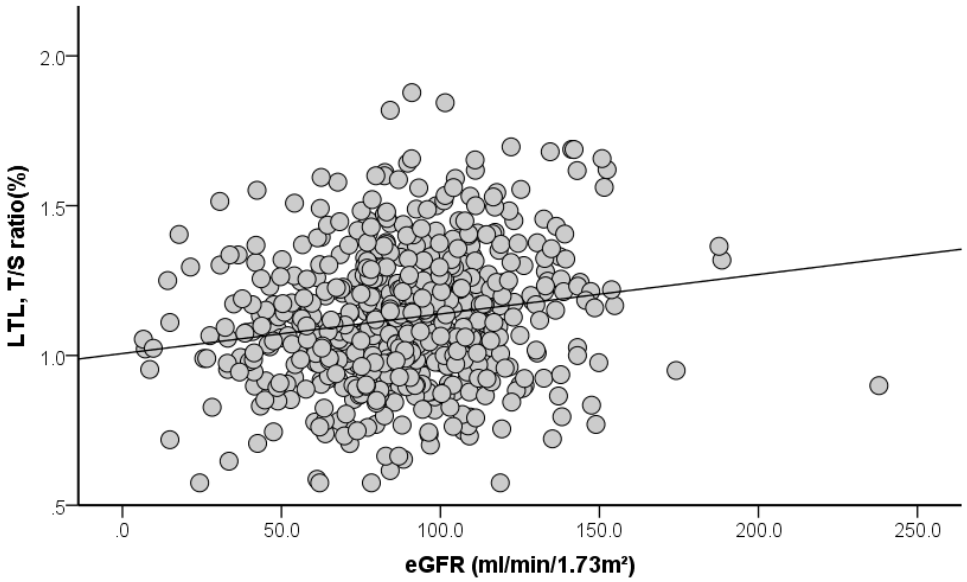


Figure 5.5 Scatter plot of predicted T/S ratios against values of eGFR.
Analysis is adjusted model for age, gender, ethnicity, diabetes type, smoking, waist circumference and circulating levels of endotoxin, insulin and C reactive protein. (R=0.17, P<0.001)

Medication use differed greatly amongst individuals groups but these differences were statistically significant only in the subgroup of people with type 1 diabetes (Table 5.3).

Table 5.3 Medications history of 630 individuals with diabetes grouped by severity of BPE score.

	Medications	Group 1	Group 2	Group 3	Group 4	p value
Entire cohort	Insulin	195(76.8%)	68(59.6%)	115(54.0%)	26(56.5%)	<0.001
	Sulfonylureas	44(17.3%)	26(22.8%)	65(30.5%)	17(37.0%)	0.003
	Biguanides	98(38.6%)	58(50.9%)	125(58.7%)	28(60.9%)	<0.001
	Thiazolidinediones	11(4.3%)	10(8.8%)	28(13.2%)	7(15.2%)	0.007
	Anti-hypertensives	123(48.4%)	67(58.8%)	149(70.3%)	38(82.6%)	<0.001
	Cholesterol lowering	116(45.7%)	71(62.3%)	157(74.1%)	36(78.3%)	<0.001
Type 2 diabetic group	Insulin	45(43.3%)	25(36.2%)	58(38.4%)	23(53.5%)	0.256
	Sulfonylureas	40(38.5%)	26(37.7%)	65(42.8%)	17(39.5%)	0.865
	Biguanides	76(73.1%)	49(71.0%)	113(73.9%)	28(65.1%)	0.713
	Thiazolidinediones	11(10.6%)	10(14.5%)	28(18.5%)	7(16.3%)	0.378
	Anti-hypertensives	83(79.8%)	48(69.6%)	115(76.2%)	36(83.7%)	0.290
	Cholesterol lowering	78(75.0%)	54(78.3%)	122(80.3%)	33(76.7%)	0.790

P values are calculated with Chi square test

There was however no association between LTL and class of medications in the whole cohort as well as in the group of people with type 2 diabetes (Table 5.4).

Table 5.4 Mean (± SE) telomere length (T/S ratio) of 630 individuals with Diabetes sorted by Medications use (binary).

	Medications	No	Yes	P value
Entire cohort	Insulin	1.16±0.02	1.16±0.03	0.996
	Sulfonylureas	1.16±0.02	1.17±0.03	0.396
	Biguanides	1.17±0.02	1.15±0.02	0.366
	Thiazolidinediones	1.16±0.02	1.16±0.04	0.928
	Anti-hypertensives	1.17±0.03	1.16±0.02	0.696
	Cholesterol lowering	1.16±0.02	1.16±0.02	0.879
Type 2 diabetic group	Insulin	1.14±0.03	1.14±0.03	0.924
	Sulfonylureas	1.13±0.03	1.15±0.02	0.400
	Biguanides	1.15±0.03	1.14±0.03	0.577
	Thiazolidinediones	1.14±0.04	1.14±0.02	0.973
	Anti-hypertensives	1.15±0.03	1.13±0.03	0.478
	Cholesterol lowering	1.14±0.03	1.14±0.03	0.991

Generalized linear model adjusted for Age, Gender, Ethnicity, Smoking, and Waist circumference

5.4 Discussion

In this study we reported for the first time an association between LTL and prevalent deeper periodontal pockets in people with diabetes. The main finding was that LTL ratio was lower when severe pocket depths were greater and that this association was independent of age, diabetes type, adiposity and circulating levels of inflammatory bio-markers. We also reported that circulating endotoxin levels, insulin resistance (HOMA-IR) and eGFR were associated with LTL in people with diabetes.

In our first study we demonstrated shorter LTL in individuals with periodontitis when compared to healthy controls, suggesting also that increased systemic inflammation and oxidative stress could account for this association. We reported increased levels of systemic inflammation/oxidative stress to be associated with both diagnosis of periodontitis and shorter LTL (528). In this study we documented that these associations are present also in individuals suffering from diabetes, a disorder characterized by a chronic state of increased inflammation and oxidative stress and we suggested the bacterial burden as possible factor accounting for this association.

This suggests that the association between inflammation and LTL is continuous, with no obvious thresholds. Indeed, a progressive reduction in LTL was observed with increasing severity of the oral disease. Current understanding of the LTL dynamic and its determinants in humans are in line with our findings. The continuous recruitment and differentiation of new inflammatory cells, associated with increased oxidative stress exposure, are thought to be the primary mechanisms causing a higher rate of LTL shortening in individuals with chronic infectious and/or inflammatory diseases (75;240).

Circulating levels of LPS were inversely associated with LTL. Higher levels of LPS commonly mark a higher infective burden, which results in an increased mobilization and recruitment of inflammatory cells from the bone marrow. This process can lead to faster telomere length attrition in HSCs due to the "end replication problem" and oxidative stress exposure (240;529), ultimately resulting in shorter LTL (113). Indeed, the inverse association between LTL and LPS was observed only in subgroups with shorter LTL compared to the gingivitis group (patients with type 2 diabetes and those with severe oral disease). Conversely, edentulous patients do not have shorter LTL compared to the control group, despite their older age and higher levels of circulating LPS. This could result from a survivor effect. The low number of patients without teeth suggests that only edentulous individuals with extremely long LTL at birth or with an extremely high activity of the telomerase in the HSC might have survived at this age, leaving therefore the edentulous population with longer LTL compared to the other two groups. The high level of LPS recorded in edentulous patient is intriguing, as the lack of teeth is likely to remove the inflammatory trigger represented by the PD bacterial aggression. However, this is not unexpected as the assay used to measure LPS in this study is not specific for oral bacteria and it is likely that old people with type 2 diabetes have multiple sources of infection (i.e. gut) which can lead to a significant increase of circulating levels of LPS.

No associations were observed between circulating levels of inflammatory markers and LTL. As in our first study, this could be due to the limited information about an individual's inflammatory burden provided by circulating markers of inflammation, particularly in subjects with diabetes and different levels of periodontal inflammation. Indeed, in these patients, levels of IL-6 and CRP are likely to be

extremely variable and affected by the activity of the underlying oral inflammatory processes (254;255) as well as of the quality of gluco-metabolic control (256). In contrast, LTL appears to be a measure of the cumulative burden of inflammation and oxidative stress over individual's life span and its dynamic is thus only marginally affected by acute changes in inflammatory burden. This might explain the lack of association between telomeres and inflammatory markers observed in the current and previous study. (530)

In our survey only people with type 2 diabetes presented a linear reduction in LTL with increasing severity of BPE scores. A number of factors could account for this finding. Firstly, a relatively low number of individuals with type 1 diabetes presented with greater scores of periodontal exposure, reducing the power of our analyses. Furthermore, people generally develop type 1 diabetes early in life. They become more accustomed and compliant to diabetes treatment than people who develops type 2 diabetes in older age (522). This might account for the increased levels of cardiometabolic risk factors and inflammatory markers recorded in the type 2 rather than type 1 diabetes group, also explaining the higher prevalence of moderate to severe periodontal pockets in participants with type 2 diabetes. Lastly, in our study cohort people with type 1 diabetes were generally younger than those with type 2. This might result in a shorter period of exposure to inflammation and periodontal inflammation. It is possible that the chronic immuno-inflammatory response related to the gingival disease did not have enough time to significantly impact on the relatively slow rate of LTL shortening in humans.

It is possible that the chronic immuno-inflammatory response related to oral disease did not have enough time to impact significantly on the relatively slow rate of

LTL shortening. Finally, the high levels of insulin detected in patients with type 2 but not in those with type 1 diabetes, could actively contribute to the telomere damage in conditions of increased oxidative stress and inflammation. Animal experiments have shown that the insulin/insulin like growth factor-1 pathway (IIP) has a crucial role in regulating life span, through fine regulation of the intracellular oxidative stress levels (531;532). Indeed, binding of insulin and insulin like growth factor-1 (IGF-1) to their receptors leads to autophosphorylation of the β-subunits of the insulin or IGF-1 receptor, recruitment of insulin receptor substrate-1/-2, and subsequently activation of mainly phosphatidylinositide 3-kinase (PI3K). The PI3K pathway activates Akt which mediates Forkhead-Box O1 (FoxO1) phosphorylation and nuclear exclusion of FoxO1. As FoxO1 regulates expression of manganese SOD (MnSOD), the exclusion of FoxO1 from the nucleus results in down-regulation of the antioxidant intracellular capacities (533;534). Therefore, an increased insulin signal results in reduced intracellular antioxidant capacities, possibly increasing susceptibility to DNA oxidative stress damage in conditions of increased oxidative stress exposure. Hyperinsulinemia is a common feature of type 2 diabetic patients as it is necessary to counteract the muscular and adipose tissue insulin resistance. However, immune-inflammatory cells maintain normal levels of insulin sensitivity in type 2 diabetes and can be hyper-stimulated by the high levels of circulating insulin. This can reduce the amount of intracellular MnSOD in inflammatory cells of type 2 diabetics, possibly increasing the DNA oxidative stress damage due to the presence of PD and explaining LTL differences between type 1 and type 2 diabetics. The reduced ability to compensate for oxidative stress damage in type 2 diabetics is supported in this study by the graded decline in LTL with increasing severity of PD observed in type 2 but not in type 1 diabetics.

A robust association was found between LTL and both insulin resistance and eGFR. These findings confirm previous evidence suggesting LTL could act as a reliable marker of future diabetic complications, especially for nephropathy (520;521). Interestingly, the presence of PD has been related to an increased risk of mortality in diabetics (particularly from diabetic nephropathy and ischemic heart disease), independently from duration of diabetes and gluco-metabolic control (535). This suggests that the presence of the oral disease and related systemic inflammatory burden are the principal factors accounting for the prognosis. As LTL has been related with a higher risk of CV mortality (514) and faster progression of diabetic nephropathy (536), our study suggests that the shortened LTL resulting from the high level of periodontal exposure of diabetes patients may be a biological pathway accounting for their higher cardiorenal mortality risk.

Our study has several strengths. Firstly, it includes a large population of people with diabetes in whom the state of oral health was determined by oral examination performed by two expert periodontists, rather than being self-reported by patient with questionnaires (524). Secondly, the detailed characterization of the cohort and the availability of several covariates potentially influencing LTL enable extensive adjustments, providing an assessment of the relative impact of each parameter on LTL. Finally, the recruitment at a single hospital reduces the potential confounding impact of geographical and ethnic variability on LTL (95;537).

Nevertheless, important limitations remain. Firstly, the cross-sectional design cannot exclude reverse causality. As previously mentioned, short telomere length has been associated with aberrant cytokine production and altered immune cell function (538). Similarly, the aetiology of PD is currently though to be based on a dysregulated

immuno-inflammatory response to local bacterial aggression (539), leading to a progressive accumulation of dental plaque biofilm (276;277). Therefore, an interesting hypothesis can be generated from current data. In a population of patients with diabetes, short LTL may mark those with higher risk of developing severe and chronic infections (i.e. PD) due to an age-related deficit or dysregulated activity of their immuno-inflammatory system. Secondly, our method of assessment of periodontal diagnosis was limited to the WHO/BPE index of prevalent periodontal pockets and treatment needs. This is not considered a clinical method of periodontitis diagnosis (as lacking of measures of gingival recession/attachment loss and radiographic assessment of alveolar bone levels) but rather a method of prevalent severity of PPDs in population surveys. Whilst in the past CPITN/BPE has been used as method of periodontal exposure, clear limitations of the index have been reported especially as it could let an underestimation of the periodontal tissue destruction. The subgroup analysis based on full mouth periodontal measures confirmed the positive discriminative value of categorizing the survey population in distinctive groups of periodontal exposure (i.e. gingivitis, moderate and severe periodontitis) and that if at all our approach might have produced an underestimation of the overall exposure to periodontitis. Thirdly, the presence of severe oral infection in patients with PD might determine a change in the relative proportion of leukocyte subpopulations in peripheral blood. As the quantitative PCR-based assay provides only the average telomere length across all leukocytes, it is impossible to exclude that this could partially account for the associations found. However, it is now well established that the high inter-individual variability in telomere length far exceeds the variations among cell types within the same individual, resulting in a high synchronization of telomere length between different cells and tissues of both healthy (88;90) and

diseased subjects (515;516). Fourth, the LPS assay used in this study measures the total amount of LPS activity in peripheral blood, without providing specific information on the possible origins of the infectious burden. As diabetics are more prone to develop serious infections than the general population (540), it is not possible to exclude that subjects with increased severity of periodontal inflammation also had other concomitant infections. Finally, while the elimination of the infectious and inflammatory stimuli can account for the lack of significant difference in LTL between the edentulous and the gingivitis groups, the low number and significantly higher age range of these patients compared to the other groups complicate interpretation of the results.

In conclusion, our study shows that in patients with diabetes, prevalent moderate to severe periodontal inflammation is associated with shorter LTL, independently from metabolic factors. This association can mark an age-related dysfunction of the immune-inflammatory system (potentially increasing the individual predisposition to chronic infections) or it can be a consequence of the higher levels of inflammatory and oxidative stress exposure related to the oral disease.

KEY POINTS

The association between chronic inflammation and LTL in patients with increased periodontal inflammation is independent from metabolic factors

The infective burden reflected by circulating levels of LPS could represent the chronic inflammatory trigger which leads to shorter LTL in PD

The cumulative inflammatory burden reflected by the amount of LTL shortening is likely to contribute to the evolution of diabetic complication in patients with PD

6 STUDY 3 – Early origins of the association between inflammatory burden and LTL: a study in young individuals.

6.1 Introduction

This study explored the early origins of the link between chronic inflammation and LTL.

Studies performed in adult populations have described negative associations between LTL, clusters of traditional CV risk factors (including dyslipidaemia, diabetes mellitus, obesity, and smoking) (541;542), as well as with subclinical (543) and clinical measures atherosclerosis (354). However, the biological basis for these associations remains unclear. Indeed, while exposure to CV risk factors can increase levels of inflammation, it is also well established that higher levels of inflammation can lead to emergence of insulin-resistance, hypertension and dyslipidaemia (173). Therefore, it is unclear whether adult associations between short LTL and CV disease are directly influenced by levels of CV risk factor exposure or it is the common pathway of inflammation that affects the risk of developing adverse cardio-metabolic patterns, CV disease and short LTL. Studies of young populations with low CV risk factor burden as well as shorter exposure to inflammatory triggers, provides the opportunity to identify early biological pathways relevant to LTL dynamics. Any association identified in this population would be less confounded by the presence of other underlying pro-inflammatory factors.

The aim of this study was to explore the early origins of the link between inflammation, LTL and CV diseases (used as model of age-related disease) seen in adults.

LTL were therefore measured in adolescents (13–16 years old) who took part in the third phase of the Ten Towns Heart Health Study. This study was originally designed to determine the early factors accounting for discrepancies in adult CV disease risk between areas of England and Wales with high and low adult CV mortality. The detailed characterization of the cohort, including measurement of traditional and novel CV risk factor as well as CV phenotype, enabled appropriate adjustment for most of the environmental factors that have previously been associated with short LTL in adults.

6.2 Specific Methods

6.2.1 Population

The Ten Town Heart Health Study is a mixed longitudinal study based on children attending a stratified random sample of one hundred primary schools in 10 towns across England and Wales (544;545). The 406 local authority districts in England and Wales were ranked on the basis of their standardised mortality ratios for all CV disease for 1979-1983, calculated for men and women aged 35-64 years. The five highest and five lowest mortality districts containing a population centre of 40000-100000 subjects were selected. The geographical distribution of the study towns is shown in figure 6.1.

Figure 6.1 Ten towns study.
Geographical distribution of study towns. Adapted from Whincup et al BMJ 1996;313:79-84

The study was structured in three different phases. The first was carried between January and July 1990 and explored whether values of blood pressure recorded in children aged 5-7.5 years differed between towns with exceptionally high and exceptionally low adult CV mortality. In 1994, a second phase of the study investigated whether patterns of CV risk factors emerged in children aged 8-11 years may account for geographic differences in CV mortality risk. In this run, a subgroup of 1287 children underwent blood sample collection. Finally, during the third phase of the study, carried out in 1998–2000, 1248 European and 90 South Asian adolescents (aged 13–16 years) were recruited from 69 secondary schools which included at least 20 pupils who participated in the earlier phases of the study. Pupils who had previously taken part were invited (average 45 pupils per school), together with a random sample of new participants (average 13 pupils per school). Information and DNA samples collected during the third phase of the project were used in a cross-sectional study design. Ethical approval was received from the local research ethics committees, and written informed consent was obtained from all participants and their parents.

6.2.2 Demographic, anthropometric and behavioural characterization

Ethnicity was defined on the basis of appearance, cross-checked with parental place of birth. Height was measured with a portable stadiometer (CMS Ltd, Camden, UK), weight with a digital electronic weighing scale (Soehnle Ltd, Murrhardt, Germany). Waist and hip circumferences were measured using standard techniques (544) and skinfold thickness was measured at four sites (triceps, biceps, subscapular and suprailiac) (546). Bioimpedance was measured with a Bodystat 500 (Bodystat, Ltd, IoM); estimates of percentage body fat were determined using the equation of

Deurenberg et al (547). Pubertal status was ascertained using a confidential self-assessment questionnaire based on Tanner pubic hair, penile and breast development scales (548). Participants provided questionnaire-based information on how their physical activity levels compared with those of their peers, with 5 grades (much less active, slightly less active, similar, more active, much more active) (549;550). This was cross-checked with information provided by the parents. A parental questionnaire sent immediately after examination provided information on infant feeding practices in the first 3 months of life (breastfed only, bottle-fed only, and mixed feeding) and on the duration of breastfeeding when applicable. Parental occupation was used to define social class in accordance with the Registrar General's (ONS) 1990 coding manual (551); head of household's social class was defined as paternal social class or (when not available) maternal social class. Cigarette smoking status was established by a questionnaire regarding current and lifelong smoking, in order to separate participants into current smokers (>=1 cigarette per day currently), past smokers (history of previous but not current cigarette smoking), and non-smokers. Salivary cotinine measurements were used to reclassify past or non-smokers as current smokers (levels >=14.1 ng/mL) (552). All assessments were made by a trained field team (four observers) who visited each town in turn. A blood sample was collected after an overnight fast for DNA extraction and measurements of traditional and novel markers of CV risk. Finally, brachial artery distensibility was measured to determine, non-invasively, the adolescent's CV phenotype.

6.2.3 Blood Samples

Blood samples were frozen (−20°C) within 6 hours of collection and transferred to a central laboratory for analysis within 2 weeks or for long-term storage

at −70°C. Total serum cholesterol and high-density lipoproteins were measured using a Hitachi 747 automated analyser (Roche Diagnostics Corp, Indianapolis, IN) at the Royal Free Hospital (553;554), while LDL was calculated using the Friedewald equation (555). Triglycerides (maximum 4.7 mmol/L) were within the limit for determining LDL (10 mmol/L) using the Friedewald equation. Plasma glucose was measured in a fluoride oxalate sample with a Falcor 600 automated analyser and serum insulin using a specific ELISA assay which does not cross-react with proinsulin (556). The assessment of insulin resistance was based on the homeostasis model assessment (HOMA) equation (glucose × insulin/22.5).

CRP was determined with a high sensitive enzyme immunoassay with DAKO reagents (557) and clottable fibrinogen was measured with the Clauss method in blood anti-coagulated with 0.109 mol/Ltrisodium citrate (9:1 vol:vol) (558). Plasma vitamin C and was measured in samples pre-treated with metaphosphoric acid at the point of collection and then snap-frozen with dry ice using high performance liquid chromatography (HPLC) (559). Plasma levels of tissue Plasminogen Activator (tPA) and von Willebrand factor (vWF) were measured with ELISAs (Biopool AB and DAKO, respectively) (560). Serum total homocysteine was measured by a modified automated assay, based on pre-column derivatisation with monobromobimane, followed by reverse phase high performance liquid chromatography with fluorescence detection (561). Serum folate levels were determined by a microbiological assay with the use of a chloramphenicol resistant strain of Lactobacillus casei (562). Each assay was performed by laboratory staff blinded to the town from which the sample was collected.

6.2.4 DNA extraction, standardization and LTL assay

The protocols used for DNA extraction, standardization and LTL assay were similar to those reported in the general methods. However, as the LTL technique used in previous studies was validated on adult samples, I tested the reproducibility of the assay for younger samples, where longer LTL were expected. Thus, I randomly selected a subset of 20 DNA samples from the cohort and I ran them in duplicate on two different days. The inter-assay coefficient of variation was 3.27%; the correlation coefficient between the average T/S ratio determined by the first and second runs was 0.93 (figure 6.2).

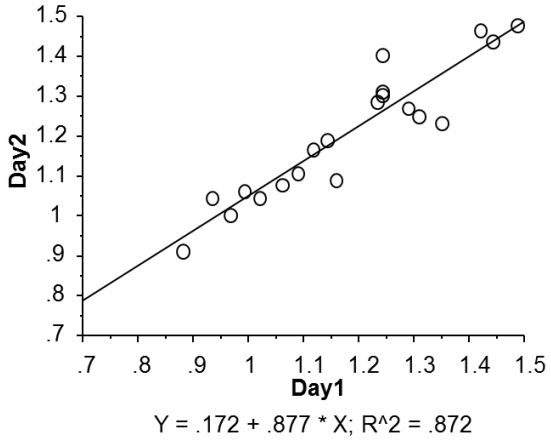


Figure 6.2 Reproducibility of the LTL assay in the Ten Towns Study.
Scatter plot reporting the strong correlation between T/S values of 20 DNA samples randomly selected from the cohort and run in duplicate on two different days.
R^2=0.872

The estimate of the coefficient of variation, though potentially an underestimate of the true coefficient of variation based on separate DNA extractions, was lower than that of several previous studies using similar methods (82;250;479), confirming the high reproducibility of the protocol also for higher values of LTL. The corresponding LTL in base pairs from the T/S ratio measured in each subject was obtained by means of the regression line resulting from comparison between T/S ratio

of our Q-PCR assay and the standard terminal restriction fragment (TRF) analysis (479).

6.2.5 Brachial artery distensibility

Brachial artery distensibility was measured using the protocol reported in the general methods.

6.2.6 Statistical Analysis

Statistical analyses were carried out using STATA/SE software (Stata/SE 10 for Windows; StataCorp LP, College Station, TX). Variables were checked for normality and log transformed where necessary. Means and standard deviations (or geometric means and standard deviations for log-transformed variables) for adiposity and blood analyses were presented unadjusted. Means and differences in LTL were adjusted for age, sex, ethnicity, and town using the regress and lincom procedures within STATA. Estimates of mean LTL by quintiles of CRP and fibrinogen adjusted for age, sex, ethnicity, and town were obtained using the same procedures. Associations between anthropometry, blood pressure, blood analytes, and LTL were examined using linear regression models adjusted for age, sex, ethnicity, and town.

6.3 Results

Anthropometric, blood, and LTL measurements were available from 1080 subjects (992 white Europeans, 73 South Asians, and 15 from other ethnicities). Measures of LTL were normally distributed (Figure 6.3) with a mean of 1.30 T/S ratio (absolute value 8494bp) and a large inter-individual variability (T/S range: 0.65 - 2.32; absolute range: 5517-13166bp).

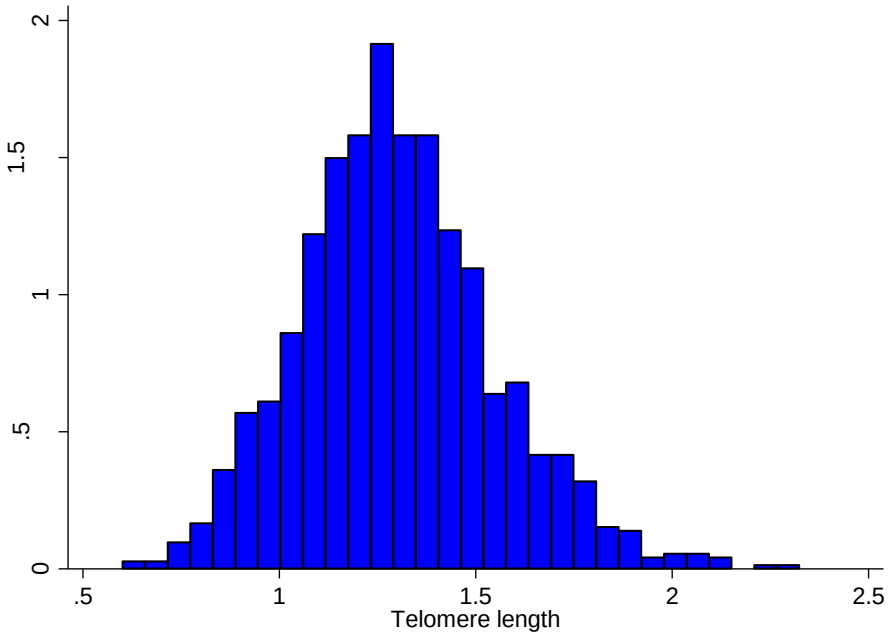


Figure 6.3 Distribution of LTL in the Ten Towns Study.
LTL were normally distributed with a mean T/S ratio of 1.30 corresponding to an absolute value 8494bp. There was a large inter-individual variability of LTL (T/S range: 0.65 - 2.32; absolute range: 5517-13166bp).

Clinical characteristics of participants are shown in Table 6.1. The same table shows the associations between each variable and LTL after adjustment for age, ethnicity, sex, and town.

Table 6.1 Characteristics of the Ten Towns population and associations with LTL

Variable (N=1080)	Geometric mean	Geometric SD	Difference in T/S ratio for one SD / Log SD(†) increase in variable (95% CI)	p (linear association)
Height (cm)	166.9	1.1	0.014 (-0.004, 0.032)	0.12
BMI (Kg/m^2)*	20.6	1.2	-0.008 (-0.023, 0.007)	0.32
Waist Circumference (cm)*	69.7	1.1	0.003 (-0.019, 0.012)	0.66
Sum of skinfolds (mm)*	46.0	1.6	-0.011 (-0.028, 0.006)	0.21
Fat mass (%)	25.9	6.5	-0.013 (-0.030, 0.004)	0.12
Systolic BP (mmHg)	120.7	13.3	0.010 (-0.006, 0.025)	0.21
Diastolic BP (mmHg)	66.9	7.2	0.012 (-0.003, 0.027)	0.11
Pulse pressure (mmHg)	53.8	11.3	0.003 (-0.013, 0.019)	0.68
Total cholesterol (mmol/L)	4.2	0.7	0.007 (-0.008, 0.023)	0.35
LDL cholesterol (mmol/L)	2.3	0.6	0.005 (-0.010, 0.020)	0.48
HDL cholesterol (mmol/L)	1.5	0.3	0.013 (-0.002, 0.028)	0.10
Triglycerides (mmol/L)*	0.9	1.4	-0.007 (-0.023, 0.008)	0.35
Glucose (mmol/L)	5.1	0.5	0.001 (-0.016, 0.017)	0.93
Insulin (mU/L)*	8.9	1.6	-0.011 (-0.026, 0.004)	0.15
Insulin resistance (HOMA)*	0.7	1.1	0.002 (-0.015, 0.018)	0.85
C-reactive protein (mg/L)*	0.2	3.3	-0.026 (-0.041, -0.011)	<0.001
Fibrinogen (g/L)*	2.5	1.2	-0.025 (-0.040, -0.010)	0.001
Von Willebrand factor (IU/dL)*	100.6	1.4	-0.011 (-0.026, 0.005)	0.18
Tissue plasminogen activ. (ng/ml)*	5.4	1.5	0.005 (-0.010, 0.020)	0.53
Homocysteine (μmol/L)*	8.0	1.3	0.001 (-0.014, 0.016)	0.88
Vitamin C (μmol/L)	49.9	23.6	-0.003 (-0.020, 0.014)	0.73
Folate (nmol/L)*	14.2	1.7	0.010 (-0.005, 0.026)	0.18

*Adjusted for sex, age, ethnicity and town. Missing values: Sum of skinfolds (n=27). *Geometric mean and geometric SD and difference per log SD change presented for log transformed variables*

A negative linear association was found between LTL and age (β=-0.016, 95% CI [-0.032 to -0.001], p=0.04), whereas there were no associations between LTL and circulating levels of traditional CV risk factors including dyslipidaemia, hypertension, insulin resistance, and obesity. However, there was a strong inverse association between LTL and circulating levels of inflammatory markers, CRP (β =-0.026, 95% CI [-0.041 to -0.011], p<0.001) and fibrinogen (β =-0.025, 95% CI [-0.040 to -0.010], p=0.001). There was a graded decline in mean LTL with increasing levels of CRP, and mean LTL was significantly shorter in subjects in the top quintile of CRP levels compared with the first quintile (mean difference T/S ratio 0.09, absolute value mean difference 408bp, p<0.001) (Figure 6.4A).

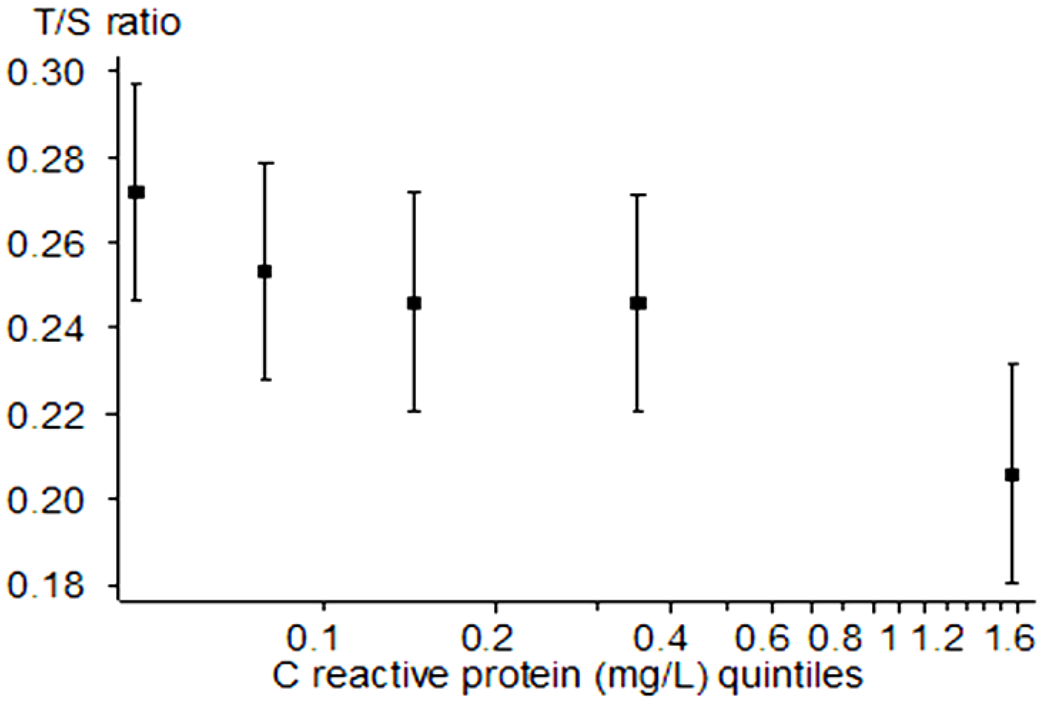


Figure 6.4A. Association between LTL and CRP in the Ten Towns Study.
Graded reduction of LTL was observed with increasing quintile of CRP. Results are adjusted for sex, age, ethnicity and town. X-axis on LTL vs CRP association is in log scale.

Similarly, for fibrinogen (Figure 6.4B), the mean LTL was significantly shorter in subjects in the top quintile compared with the first quintile (mean difference T/S ratio 0.07, absolute value mean difference 331bp, *p*=0.003).

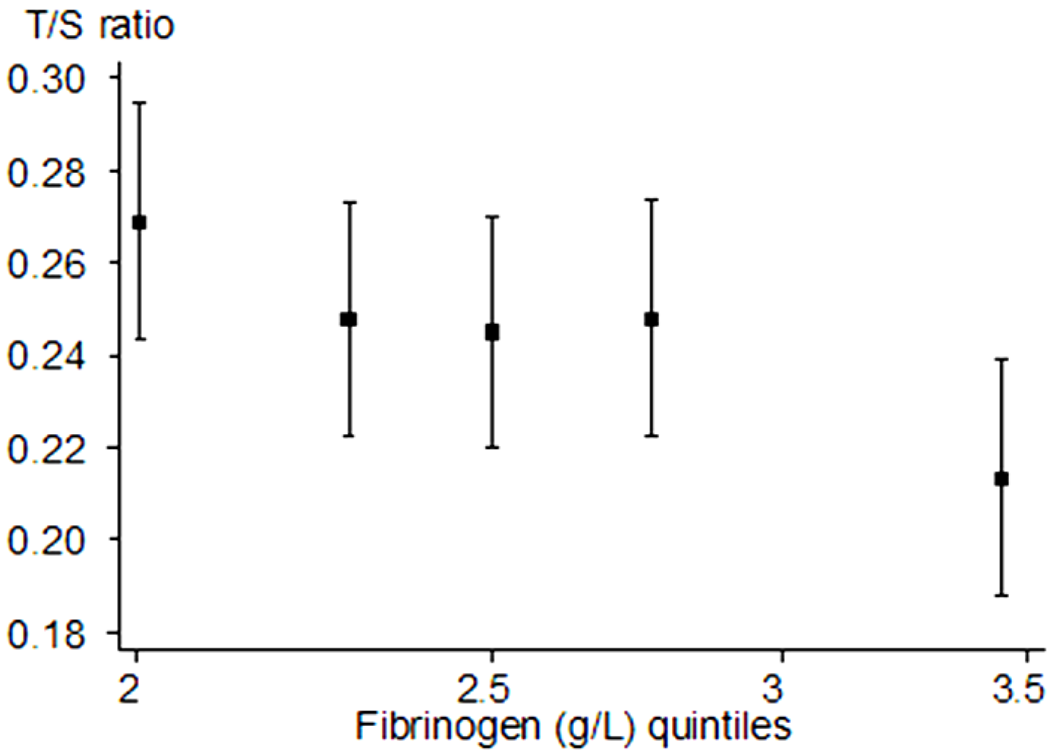


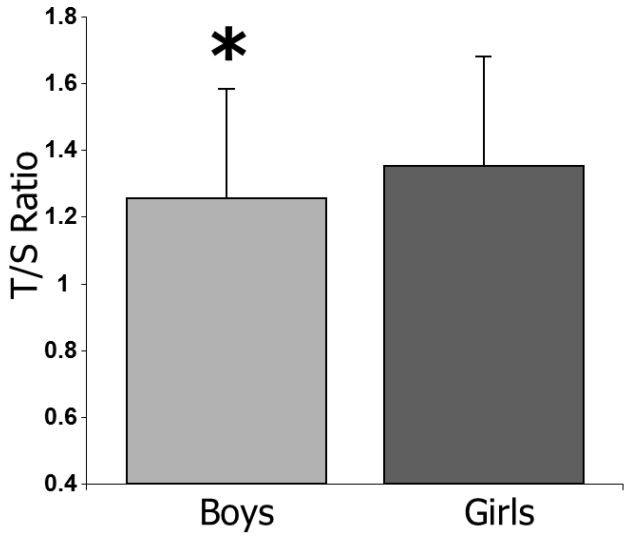
Figure 6.5B Association between LTL and fibrinogen in the Ten Towns Study.
Graded reduction of LTL was observed with increasing quintile of Fibrinogen. Results are adjusted for sex, age, ethnicity and town.

CRP and fibrinogen levels were strongly associated with each other (*r*=0.6; *p*<0.001). The associations between CRP, fibrinogen, and LTL were not mutually adjusted because these two strongly intercorrelated variables are likely to represent the same biological pathway. No sex interaction was found for the associations between LTL and inflammatory markers and no differences were found between groups of towns with high and low adult CV mortality.

Males had significantly shorter LTL than females (mean difference T/S ratio 0.03, absolute value mean difference 138 bp, *p*=0.03) (Figure 6.5A), whereas subjects

of South Asian ethnicity showed longer LTL than white Europeans (mean difference T/S ratio 0.10, absolute value mean difference 458 bp,*p*=0.01) (Figure 6.5B).

A



B

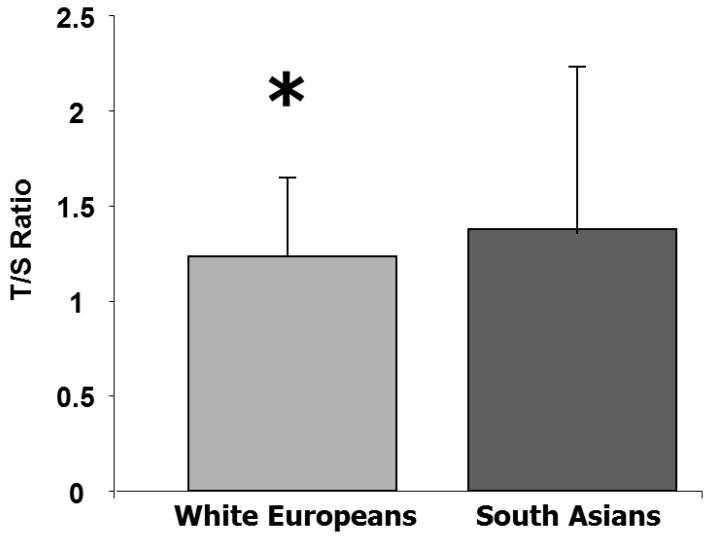


Figure 6.6 Gender and ethnic gap of LTL in the Ten Towns Study.
Boys had significantly shorter LTL compared to girls (p=0.03) (A). Furthermore, South Asian had longer LTL compared to White European participants (p=0.01)(B).

Current and previous smokers had shorter LTL than non-smokers (*P*=0.06 for a difference between the 3 smoking groups) with and without correction for cotinine levels. Social class, levels of physical activity and history of breast feeding were not

related with LTL (Table 6.2). Furthermore, no differences in LTL were observed between different Tanner stages.

Table 6.2 Difference of LTL for social class, breast feeding, levels of physical activity and smoking status

Variables	Categories	N	Mean	(95% CI)	p (Diff.)
Social Class	1	101	1.32	(1.27, 1.37)	0.53
	2	288	1.32	(1.29, 1.35)	
	3	112	1.32	(1.28, 1.37)	
	4	127	1.27	(1.23, 1.31)	
	5	43	1.28	(1.21, 1.36)	
	6	295	1.30	(1.28, 1.33)	
	7	58	1.29	(1.22, 1.36)	
Breast Feeding (during first 3 months)	Breast fed	405	1.31	(1.28, 1.33)	0.92
	Bottle fed	375	1.30	(1.27, 1.33)	
	Mixture	230	1.31	(1.27, 1.34)	
Levels of Physical Activity (1 = Low)	1	47	1.35	(1.28, 1.42)	0.52
	2	146	1.33	(1.28, 1.37)	
	3	434	1.30	(1.28, 1.32)	
	4	285	1.30	(1.27, 1.33)	
	5	138	1.29	(1.25, 1.33)	
Smoking status*	Current	260	1.29	(1.26, 1.33)	0.06
	Past	367	1.28	(1.26, 1.31)	
	Never	447	1.32	(1.30, 1.34)	

All analyses adjusted for sex, age ethnicity and town. Smoking status difference is unsorted

Consequently, the strength of the inverse association between LTL and inflammatory markers was not affected by adjustments for behavioural factors (physical activity and smoking status), metabolic factors (body mass index,

parameters of metabolic syndrome), circulating levels of antioxidant molecules (ie, folate, vitamin C), and socioeconomic and puberty status (Tanner stage) (Table 6.3).

Table 6.3 Associations between LTL, CRP and Fibrinogen adjusted for other factors

Variable	Additionally adjusted for	Difference in T/S ratio for log SD increase variable (95% CI)	p (linear assoc)
CRP (mg/L)	No additional adjustments	-0.026 (-0.041, -0.011)	<0.001
	Physical activity (5 level variable)	-0.028 (-0.043, -0.012)	<0.001
	Smoking status corrected for cotinine	-0.026 (-0.041, -0.011)	<0.001
	Folate, Vitamin C	-0.026 (-0.041, -0.011)	<0.001
	BMI	-0.026 (-0.042, -0.010)	0.001
	WC[§], triglycerides, HDL, SBP[¶], Glucose	-0.026 (-0.042, -0.010)	0.001
	All of the above	-0.026 (-0.042, -0.010)	0.002
Fibrinogen (g/L)	No additional adjustments	-0.025 (-0.040, -0.010)	0.001
	Physical activity (5 level variable)	-0.025 (-0.040, -0.010)	0.001
	Smoking status corrected for cotinine	-0.025 (-0.040, -0.010)	0.001
	Folate, Vitamin C	-0.026 (-0.041, -0.011)	<0.001
	BMI	-0.025 (-0.041, -0.009)	0.002
	WC[§], triglycerides, HDL, SBP[¶], Glucose	-0.024 (-0.040, -0.009)	0.002
	All of the above	-0.023 (-0.039, -0.007)	0.006

All differences adjusted for sex, age, ethnicity and town. § WC: Waist Circumference, ¶ SBP: Systolic Blood Pressure.

The sex and ethnic differences in LTL were not affected by adjustment for anthropometric parameters, Tanner stage, smoking status, levels of physical activity, socioeconomic status, and CV risk factors.

A linear negative association was found between LTL and brachial artery distensibility (Table 6.4), which remained also after multiple adjustments for levels of traditional and novel CV risk factors.

Table 6.4 Association between Telomere length (T/S ratio) and distensibility

	N	Difference in T/S ratio for SD increase Distensibility (95% CI)	p (linear association)
Brachial artery Distensibility	260	-0.046 (-0.085, -0.007)	0.02

Adjusted for sex, age, ethnicity, pulse pressure and town (4 towns)

All analyses were repeated in the white European participants alone (Table 6.5), as well as in females and males separately (Table 6.6), and the results were not materially affected (see next pages).

Table 6.5 Associations between Telomere length (T/S ratio) and other variables – for white Europeans only

White Europeans Variable (N=992)	Mean/ geometric mean	SD/ geometric SD	Difference in T/S ratio for one SD / log SD† increase in variable (95% CI)		p(linear assocs)
Age (years)	15.1	0.6	-0.016	(-0.033, 0.000)	0.06
Height (cm)	167.5	8.5	0.015	(-0.004, 0.033)	0.12
BMI (kg/m^2)*	20.7	1.2	-0.006	(-0.022, 0.010)	0.47
Waist circumference (cm)*	69.8	1.1	-0.004	(-0.020, 0.012)	0.64
Sum of skinfolds (mm)*	46.2	1.5	-0.008	(-0.025, 0.010)	0.40
Fat mass %	25.9	6.5	-0.013	(-0.031, 0.005)	0.15
Systolic BP	121.0	13.4	0.010	(-0.006, 0.026)	0.22
Diastolic BP	67.0	7.2	0.012	(-0.004, 0.027)	0.14
Pulse pressure	54.0	11.3	0.004	(-0.012, 0.021)	0.62
Cholesterol (mmol/L)	4.2	0.7	0.009	(-0.007, 0.025)	0.29
LDL cholesterol (mmol/L)	2.3	0.6	0.007	(-0.009, 0.022)	0.41
HDL cholesterol (mmol/L)	1.5	0.3	0.014	(-0.002, 0.030)	0.08
Triglyceride (mmol/L)*	0.9	1.4	-0.008	(-0.025, 0.008)	0.32
Glucose (mmol/L)	5.0	0.5	0.001	(-0.016, 0.019)	0.88
Insulin (mU/L)*	8.9	1.6	-0.010	(-0.025, 0.006)	0.23
Insulin resistance (HOMA)*	0.7	1.1	0.002	(-0.015, 0.018)	0.86
C-reactive protein (mg/L)*	0.2	3.3	-0.022	(-0.038, -0.007)	0.01
Von Willebrand factor (IU/dL)*	99.9	1.4	-0.011	(-0.027, 0.006)	0.20
Tissue plasminogen activator (ng/mL)*	5.3	1.5	0.006	(-0.010, 0.021)	0.49
Homocysteine (µmol/L)*	8.0	1.3	0.001	(-0.014, 0.017)	0.87
Fibrinogen (g/L)*	2.5	1.2	-0.023	(-0.039, -0.007)	0.005
Vitamin C (µmol/L)	51.6	23.1	-0.001	(-0.019, 0.016)	0.87
Folate (nmol/L)*	14.3	1.7	0.010	(-0.006, 0.026)	0.23

** Geometric mean and geometric SD and difference per log SD change presented for log transformed variables*
Adjusted for sex, age and town
Missing values: Sum of skinfolds (n=25)

Table 6.6 Associations between Telomere length (T/S ratio) and other variables by gender

	Boys			Girls			
Variable	Difference in T/S ratio for one SD / log SD† increase in variable (95% CI)		p(linear assocs)	Difference in T/S ratio for one SD / log SD† increase in variable (95%		p(linear assocs)	p (gender interaction)
Height (cm)	0.005	(-0.018, 0.027)	0.69	0.017	(-0.006, 0.041)	0.14	0.82
BMI (kg/m^2)*	-0.014	(-0.034, 0.006)	0.17	0.002	(-0.021, 0.024)	0.88	0.45
Waist circumference (cm)*	-0.008	(-0.028, 0.013)	0.46	0.003	(-0.019, 0.026)	0.77	0.67
Sum of skinfolds (mm)*	-0.013	(-0.033, 0.007)	0.21	-0.001	(-0.024, 0.022)	0.93	0.40
Fat mass %	-0.012	(-0.032, 0.008)	0.24	-0.006	(-0.028, 0.017)	0.63	0.72
Systolic BP	0.005	(-0.016, 0.026)	0.64	0.015	(-0.008, 0.037)	0.20	0.63
Diastolic BP	0.015	(-0.005, 0.036)	0.13	0.008	(-0.014, 0.030)	0.48	0.70
Pulse pressure	-0.004	(-0.025, 0.016)	0.67	0.012	(-0.010, 0.034)	0.29	0.42
Cholesterol (mmol/L)	0.009	(-0.011, 0.030)	0.39	0.007	(-0.016, 0.030)	0.54	0.85
LDL cholesterol (mmol/L)	0.009	(-0.011, 0.029)	0.38	0.004	(-0.019, 0.026)	0.75	0.83
HDL cholesterol (mmol/L)	0.009	(-0.011, 0.029)	0.39	0.016	(-0.006, 0.039)	0.15	0.36
Triglyceride (mmol/L)*	-0.004	(-0.025, 0.017)	0.71	-0.013	(-0.036, 0.011)	0.29	0.66
Glucose (mmol/L)	-0.015	(-0.037, 0.007)	0.19	0.019	(-0.006, 0.043)	0.13	0.00
Insulin (mU/L)*	-0.010	(-0.030, 0.010)	0.34	-0.013	(-0.035, 0.009)	0.25	0.95
Insulin resistance (HOMA)*	-0.013	(-0.035, 0.008)	0.23	0.018	(-0.006, 0.041)	0.14	0.01
C-reactive protein (mg/L)*	-0.020	(-0.040, 0.000)	0.05	-0.032	(-0.055, -0.010)	0.01	0.61
Von Willebrand factor (IU/dL)*	-0.016	(-0.036, 0.004)	0.11	0.001	(-0.021, 0.023)	0.90	0.17
Tissue plasminogen activator (ng/mL)*	0.014	(-0.007, 0.034)	0.19	-0.005	(-0.028, 0.017)	0.64	0.15
Homocysteine (µmol/L)*	-0.002	(-0.023, 0.018)	0.81	0.009	(-0.014, 0.032)	0.43	0.53
Fibrinogen (g/L)*	-0.014	(-0.034, 0.006)	0.17	-0.034	(-0.057, -0.012)	0.00	0.33
Vitamin C (µmol/L)	0.002	(-0.020, 0.025)	0.84	-0.009	(-0.035, 0.016)	0.46	0.19
Folate (nmol/L)*	0.006	(-0.014, 0.026)	0.56	0.012	(-0.011, 0.034)	0.31	0.70

** Geometric mean and geometric SD and difference per log SD change presented for log transformed variables*
Adjusted for age, ethnicity and town
Missing values: Sum of skinfolds (n=27)

6.4 Discussion

This was the first study to report an association between LTL, inflammatory markers and CV phenotype in a young and well-characterized cohort of healthy adolescents. The key finding is that LTL was not related to a range of traditional CV risk factors but was inversely related to two measures of inflammation, CRP and fibrinogen, both of which have been associated with adverse CV outcomes (563-579). However, an unexpected negative relationship between LTL and brachial artery distensibility was detected, suggesting that adolescents with longer LTL have an increased burden of CV damage. Furthermore, this study showed, for the first time, that the sex difference in LTL, which has been described in most adult studies, is already present early after puberty (at 13–16 years of age) and was not materially affected by adjustment for CV risk factors, Tanner stage, or other environmental or behavioral factors, suggesting that the contribution of estrogen and environmental exposure to this difference was small. Finally, South Asians had longer LTL compared to Europeans, despite their higher CV mortality recorded in adult life.

Two recent studies have measured LTL in children and adolescents. In a cross-sectional case control study including children of 2–17 years old, Buxton et al (580) reported significantly shorter LTL in obese children compared to their lean peers. Our findings differ from with those of Buxton and are in agreement with Zhu et al who did not document associations between biochemical and anthropometric measures of adiposity and LTL in a cohort of 667 young adolescents (581). Our study as that of Zhu et al., however, was not designed to explore an association between LTL and adiposity. Indeed, <5% of children in both populations were obese and lack of power may explain the negative findings. The most important difference in our data

compared with those of Buxton et al and Zhu et al is that, while both of these previous studies measured LTL in children and young adolescents, neither examined the impact of inflammation or other CV risk factors on LTL.

The relationship between CV risk factors, inflammatory markers, and LTL had been previously explored only in older cohorts (62;63). Analysis of the placebo arm of a randomized controlled trial found that short LTL was a predictor of early myocardial infarction in adulthood, although this relationship was not explained by correlation between LTL and established CV risk factors (61). Our current findings suggested that the relationship between inflammatory burden and LTL begins early in life and persists for physiological levels of inflammation. It is now clear that atherosclerosis is an aging-related systemic disease which begins from childhood (582;583) and is largely driven by a chronic inflammatory and oxidative stress exposure (332;584). Accordingly, it has been suggested that the shortened LTL often observed in adult patients who suffer from atherosclerosis may be the result of a continuous stimulus to the recruitment, differentiation and replication of new inflammatory cells as well as a higher level oxidative stress mediated damage to the telomere sequence (75;240). While the inverse relationship between inflammatory markers and LTL recorded in our study strongly support this hypothesis, the finding that adolescents with longer LTL had a lower brachial distensibility is unexplained. Indeed, adolescents with higher brachial artery distensibility (lower level of vascular damage) had shorter LTL (normally associated with higher risk of CV disease). Several factors could account for this finding. It is possible that adolescents with increased CV damage due to higher inflammation tend to mobilize a higher number of endothelial progenitor cells from the bone marrow in the attempt to repair the vascular

injury. Consequently, at 13-16 year old, subjects exposed to higher levels of inflammation during childhood could have shorter LTL but better arterial distensibility because of the hyperactivation of vascular repair mechanisms (585). A recent study from Charakida et al demonstrated that exposure to CV risk factors during the first decade of life is associated with a better vascular phenotype at 11 years old (586). The hyper-compensation to the vascular damage in early life could have significant costs for the future. Indeed, a higher mobilization of endothelial progenitor cells is sustained by an increased number of HSC replications, their haematopoietic precursors (112). This will further increase the rate of telomere attrition in HSC/endothelial progenitor cells, over and above the contribution of the inflammatory burden. As production and mobilization of endothelial progenitor cells is dependent on telomere length (587), subjects with faster rate of telomere length attrition during early life could lack effective vascular repair mechanisms during adulthood, as they will be those with shorter telomere length. At this stage, the lifelong exposure to inflammation may have caused high levels of vascular damage coupled with accumulation of HSC/endothelial progenitor cells with short telomere length. Therefore, the increased burden of vascular damage cannot be properly repaired due to the reduced ability to mobilize endothelial progenitor cells from the bone marrow. These mechanisms would result in the emergence of the expected association between LTL and measures of vascular remodelling, normally described in adult studies.

The relationship between CRP and fibrinogen with LTL was observed at levels below those associated with CV risk elevation in adults (574). This is remarkable, as it suggests that even physiological variations in inflammatory states could have an

effect on LTL biology in the young. This is supported by a recent, large meta-analysis which demonstrated a continuous relationship between CRP and CV outcome, even after adjustment for traditional CV risk factors.

The inflammatory imprinting on LTL, not explained by levels of environmental exposure (socioeconomic class, levels of physical activity, smoking status, etc.), suggests that heritable factors could represent the earliest determinants of the association between short LTL and inflammation. In the general population, these factors could set different levels of inflammatory response generated by a common environmental burden, in this way affecting the rate of LTL shortening. An additional interpretation of this finding is that peri- and early post-natal exposure to infections might cause shorter LTL in adolescents with a higher inflammatory burden. Indeed, an ubiquitous and repeated driver of early life inflammation is infection. Childhood infections are commoner and more severe in boys, who have shorter LTL (588). Furthermore, chronic infections, such as cytomegalovirus (CMV), are associated with adult CV disease (589), and also result in shorter LTL, by increasing T cell proliferation (515;590). Therefore, infection can determine increase inflammatory burden and proliferation of immune cells, possibly accounting for the described association between inflammatory markers and LTL. While this represents an intriguing hypothesis, it is unlikely that, at this early stage of life, common infections might have had a major impact on the rate of LTL shortening. Indeed, among healthy children and young adolescents, the most common infections normally present acute pathologies. As suggested in our first study, short lasting inflammatory responses hardly impact on LTL, even when the infective burden is much more severe and longer than that recorded in young adolescents. Furthermore, CMV infection has been

shown to short telomere length in middle age populations (515;590), where the infective burden and its consequences on LTL dynamic is likely to be much greater than those affecting young populations. Finally, CMV infection shortens telomere length in small subgroups of inflammatory cells (T-cells) (515;590), while qPCR measures the average telomere length across the whole set of peripheral leukocytes. It is therefore unlikely that CMV infection could provide a substantial contribution to average LTL measured at such young age.

LTL was shorter in male than female subjects and this difference was not affected by adjustment for Tanner pubertal stage. An independent influence of gender on LTL has been reported in most adult studies (62;87;95), while in newborns this difference does not exist (89). Our current findings fill the gap between adulthood and birth, showing that the gender gap in LTL is already present during early adolescence. Zhu et al recently reported similar results, showing longer LTL in adolescent females compared with males using a smaller cohort of healthy white and black adolescents (581). The longer LTL recorded in female as opposed to male subjects has previously been attributed to the anti-inflammatory and anti-oxidative effects of endogenous oestrogens (591). A short term oestrogen exposure could potentially explain current finding, as most of the adolescents in the study had already completed their pubertal maturation and the difference in LTL observed was relatively modest. However, at 13 to 16 years of age, the exposure to sexual hormones is likely to be limited to 3-4 years, while the gender difference in LTL recorded in our population was similar to that reported in middle-age populations with much longer oestrogen burden (87;95). Furthermore, the sex difference in LTL is not influenced by adjustments for pubertal stage and no gender interaction was found in the relationship between LTL and

inflammation. This, together with the longitudinal findings from the Bogalusa Heart Study, which did not find differences in the rate of telomere shortening between females and males (87), suggests that other factors may account for the gender gap in LTL. Robust correlations in LTL have been described between fathers and daughters, between mothers and sons and daughters, and among siblings (97). An X-linked inheritance of LTL is the most probable explanation for these findings, suggesting that gene variance on the X chromosome exerts an important influence on telomere length dynamics. Our current findings support this hypothesis and further suggest that factors accounting for this X-linked heritance are likely to exert their effects during the pre-pubertal period. The gender gap in LTL defined in early adolescence seems to be slightly amplified by the oestrogen exposure during reproductive age and could account, at least in part, for the sexual dimorphism of the adult association between early markers of atherosclerosis and LTL (353;543).

Considering their higher adult CV risk compared with White Europeans (592), it was unexpected to find longer LTL in South Asian subjects. This is the first study where LTL has been compared in young and healthy individuals of these two ethnic groups. Hunt et al have shown that ethnicity is an important determinant of LTL (95) but, similarly to our results, they found that African Americans had longer LTL compared to White Americans, despite their higher adult CV risk. They explained this difference by calculating the hypothetical rate of telomere shortening in their population (starting from the measures of LTL recorded in different individuals with different ages). Despite the limitations of this analysis, they showed that the rate of telomere attrition across their cohort was higher in Afro-Americans than in their peers. Recently, Aviv et al confirmed these results by measuring the longitudinal rate

of LTL change in White and African Americans who took part to the Bogalusa Heart Study (87). The narrow age range of the population, the relatively low number of South Asian subjects and the lack of serial samples available for the LTL measure did not permit similar analyses in the present study population, but a similar mechanism could explain the observed findings.

Social class was not associated with LTL. It has been hypothesized that more socio-economically deprived individuals age faster and, thus, have shorter telomeres than their more affluent counterparts (593). While a positive but weak association between white LTL and socio-economic status has previously been reported by Cherkas et al in a large heterogeneous sample of females (594), Adams et al could not confirm this finding in a study involving 318 individuals from a homogeneous birth cohort and Woo et al reported an inverse association between social status and LTL (595). The young age of the participants in our study excluded the possibility that several co-morbidities commonly found in lower social classes (e.g. obesity and smoking) may have a significant impact on LTL. This enables us to study the possible relationship between social class and LTL in a less confounded population. Therefore, the results of this and previous studies suggest that there may be little or no influence of socio-economic status on LTL.

There was no relationship between levels of physical activity and LTL in the whole cohort. These results are in contrast with previous studies reporting longer LTL in adults with higher levels of physical activity (596;597). In his cohort of adolescents, Zhu et al. found an association between LTL and vigorous levels of physical activity, but this association was present only in females and not males (581). It has been suggested that the association between longer LTL and increased physical

activity level may be mediated through an overall diminished burden of oxidative stress and inflammation in adults (581;597). Although this is a possibility, our results suggest that regulation of LTL mediated by inflammation is relatively independent from levels of physical activity in adolescence. Exercise helps to maintain energy balance and to reduce obesity and insulin resistance (598;598). These factors, in the long term, may influence on LTL dynamics by influencing levels of oxidative stress and inflammation (599;600). However, obesity and insulin resistance had a marginal impact on LTL measurements in this cohort. It is not surprising, therefore, that levels of physical activity also had a limited impact on LTL in our population.

The Ten Towns Health Heart Study represents a unique cohort in which several aspects of the LTL biology and its relationship with environmental and behavioural factors could be explored. For example, the impact of a long term environmental exposure, which is hard to quantify in adult studies, is minimized. This has potential to identify the initial and prolonged biological pathways which account for the adult association between short LTL and age-related diseases. Furthermore, the measurement of several covariates potentially influencing LTL, enabled extensive adjustments, providing a comprehensive view on the relative impact of each parameter on LTL.

Our study had some limitations. Although it is the first report to describe longer LTL in young South Asian adolescents compared to their White European counterparts, the biological meaning of this difference remains to be fully explored. Indeed, the recruited population included only a low number of South Asian adolescents and there is no evidence in the literature to support the assumption that LTL is a predictor of morbidity and mortality for age-related disease across different

ethnic groups. The cross-sectional design of the study did not permit inferring causality from the inverse association between inflammatory markers and LTL. However, it is very unlikely that LTL contributed to increased CRP and fibrinogen in this population. The presence of critically short telomeres induces a persistent activation of the DNA damage response and this has been associated with an increased production of pro-inflammatory cytokines in cultured human cells (i.e. IL-6) (242). While this mechanism could partially account for the association between inflammatory markers and LTL recorded in adult studies, the age of our cohort and the consequent longer LTL compared to adult population make the activation of the DNA damage response triggered by critically short telomeres extremely unlikely. This suggests that reverse causality is not the explanation for our results. The high inter-individual variability of LTL could have reduced the power to detect associations with CV risk factors, while the small range and young age of our population reduced the capacity to discriminate long term effects, for example, the impact of smoking status and/or obesity. This might explain the lack of certain associations compared to other reports. On the other hand, these characteristics suggest that the primary effects of CV risk factors on LTL are probably associated with the long term inflammatory burden, which begins in early adolescence.

In conclusion, our study showed that, while clinical problems from age-related diseases do not usually appear before advanced age, an association between short LTL and inflammatory markers in early adolescence may represent a possible pathophysiological pathway accounting for the relationship between short LTL and higher morbidity and mortality risk seen in adult studies. Levels of exposure to environmental and behavioural factors did not appear to contribute importantly to this

pathophysiological process in youth. As LTL records long term inflammatory burden, it could be a useful biomarker to reveal the cumulative burden of inflammation even before puberty.

KEY POINTS

● The association between inflammation and LTL:

A. Emerges from young ages

B. Remains independent from cardiovascular risk factors, metabolic and behavioural factors

● The association between LTL and cardiovascular phenotype:

A. Is inverted compared to adult studies

B. Replicates the direction of the association between cardiovascular risk factor and vascular phenotypes in the ALSPAC (a population of similar age)

C. Suggests that, in the youth, repair mechanisms can efficiently counteract the evolution of age-related diseases (i.e. vascular damage)

7 STUDY 4 - A longitudinal study of the association between rate of telomere shortening and CV damage in a large british birth cohort

The first three studies described: 1) the association between LTL and chronic inflammation, 2) its determinants and modification by metabolic factors, and 3) its origin in the young. The relevance of these pathways in the evolution of age-related diseases, however, remains largely unexplored. To answer to this question, the fourth study of this thesis explored the relationship between longitudinal variation of LTL and markers of age-related disease evolution. As a model of age-related disease we used CVD because:

1. It is a chronic inflammatory disease where inflammation is in the causal pathway for disease initiation and evolution
2. It is common in the general population
3. The subclinical phases of disease evolution can be easily quantified using non-invasive techniques applicable to large populations
4. Cross-sectional studies have already documented strong associations between LTL and CV morbidity and mortality

7.1 Introduction

The most striking feature of LTL is that it is highly variable among individuals, even after adjustment for age. Three potential causes of this variability are: (i) high inter-individual variations in telomere length at birth (89;104;601;602); (ii) high inter-individual variations in telomere attrition after birth (66;87;117;601); and (iii) variations that are attributed to the techniques used to measure telomere length. At birth, inter-individual variations in LTL amount to as much as 4 kb (89;104); during adulthood, the variations in length may be as wide as or wider than those at birth

(62;63;66;67;95;97). This considerable inter-individual variation greatly confounds the reported cross-sectional associations between short LTL and increased risk of age-related diseases. It is not known whether these associations are due to a greater rate of telomere shortening through life or due to an inherited short telomere length. This is an important issue as the identification of factors which influence the rate of telomere shortening may improve prevention of age-related disease and increase the chance of healthy aging.

Longitudinal studies assessing LTL dynamics provide a better opportunity than cross-sectional analysis to study the mechanisms linking age-related diseases to LTL. However, these investigations are constrained by two major and related limitations. The first has to do with the availability of samples, and the second to the ability of current methodology to detect small changes in telomere lengths over a limited period of follow up. Very few repositories have sequential samples of leukocyte DNA (blood or buffy coats) that have been collected longitudinally from the same individuals. Furthermore, even when such samples are available, differences in the storage and DNA extraction methods between baseline and follow up visits can drastically impact on the final results. For example, using the Southern Blot technique, the results depend on the size of the TRF, and suboptimal procedures of DNA storing which causes non-physiological breaks in the telomere region can lead to the progressive accumulation of short TRF (e.g. LTL) in the sample. Assuming that two samples have been collected within an interval of few years, the artefact due to DNA degradation will be more evident in the baseline than in the follow up sample. Furthermore, procedures to extract the DNA can drastically affect longitudinal LTL assay preformed using real time PCR. For instance, the use of two different methods to extract the DNA can lead to variable levels of protein contamination, differently

affecting the amplification efficiency of the baseline and follow up PCR analyses. The second main limitation of longitudinal LTL analysis relies on a sufficient long follow-up period to allow detection of a meaningful impact of genetic and environmental factors, and perhaps therapeutic interventions, on LTL attrition. This limitation is due to the relatively low accuracy of the current techniques used to measure LTL when compared to the slow LTL dynamics. For example, assuming one individual has LTL of 7kb. If we use a LTL assay with an inter-assay coefficient of variation of only 4%, we accept an error in repeated LTL measurements equivalent to 280b. If the same individual has a rate of LTL attrition of 80b/years, at least 3 years of follow up are necessary to have confidence that the detected changes in LTL may be due to the impact of genetic or environmental factors rather than to the intrinsic limitations of the telomere assay. In reality, the coefficient of variation of the methods that measure telomere length may considerably exceed the 4% value. Therefore, prolonged follow-up periods and large cohorts will be required to attain credible inter-individual variations in telomere attrition rates.

7.1.1 Surrogate measures of disease evolution

Beyond the limitation of current LTL assays, most of the studies that analysed the association between LTL and age-related diseases have adopted case-control experimental designs (61;63;603;604). This limits the ability to explore the role of LTL dynamics in the evolution of age-related diseases. Indeed, once age-related diseases become clinically manifest, they are normally in advanced stages, and only limited information can be recovered on the processes involved in their early evolution. Over the last 20 years, however, several techniques have been developed to follow the preclinical phases of age-related diseases. This has opened up a new field

of ‘medical epidemiology’ in which early life influences can be studied on objective measures of disease evolution and the effects of treatments determined. Atherosclerosis represents the most remarkable example of the utility of surrogate markers in understanding the mechanisms involved in disease evolution.

7.1.2 The relationship between vascular remodelling, aging, inflammation and LTL

cIMT has been considered a sensitive and reproducible marker of vascular remodelling, helping to inform on factors and mechanisms involved in the evolution of this process (605). The use of cIMT has enabled the demonstration that a physiological process of vascular remodelling occurs with aging, over and above the contribution of common CV risk factors. Indeed, values of cIMT increase naturally with aging (606). Although the range of factors that contribute to this physiological process of vascular remodelling remains unclear, increased exposure to inflammation and oxidative stress found in the elderly may well play a crucial role (329;332). Increased levels of oxidative stress within the vascular sub-endothelial space determine progressive destruction of elastin fibres accompanied by accumulation of fibrotic tissue. This process results in a progressive dilation of the vessel with reduced elasticity of the vascular wall, commonly observed with aging (607). Furthermore, the increased exposure to oxidative stress increases the expression of adhesion molecules on endothelial cells, promoting recruitment of inflammatory cells in the sub-endothelial space and further increasing the oxidative stress damage to local tissues (332;584). These processes may lead to a progressive increase of the thickness of the intima-media layer which is commonly described in the elderly (608). Growing biological evidence supports a role for inflammation and oxidative stress in the evolution of the age-related vascular remodelling (607-610). A pro-inflammatory shift

in vascular gene expression profile, including an up regulation of inflammatory cytokines, chemokines, adhesion molecules, and inducible nitric oxide synthase has been described both in rodents and in primates during normal healthy aging (611-617). Intriguingly, similar pathways are considered central to the evolution of atherosclerosis. Indeed, an aging increase in the prevalence of atherosclerotic plaques has been described in populations free of overt CV risk factors (618;619).

Few studies have explored the relationship between cellular aging pathways and cIMT. O'Donnell et al have demonstrated an inverse association between LTL and IMT of the internal carotid artery (ICA), suggesting that subjects with shorter LTL are those with increased risk of atherosclerosis (543). Similar results were obtained by Benetos et al in a population of patients with hypertension (60), as well as by Panayiotou et al (353). The former study, not only described an inverse association between LTL and cIMT, but also between LTL and the total area of the vessel occupied by atherosclerotic plaques. This suggests that LTL biology could be involved in the evolution of vascular remodelling. However, an important limitation of these reports was their cross-sectional design, which did not allow inference of causality on the association between LTL and markers of CV remodelling. Furthermore, the lack of longitudinal measurements of LTL made it impossible to understand whether the potential influence of LTL biology on the process of vascular remodelling depends on the rate of LTL attrition or on maintenance of inherited short LTL through lifespan.

The aim of this study was to determine whether rate of LTL shortening over 10 years predicted cardiac and vascular phenotypes independently from established CV risk factors.

cIMT was used as a marker of age-related disease and lifelong inflammatory burden. The association between changes of LTL over 10 years, CV risk factor burden and several measures of CV remodelling were investigated in the National Survey of Health and Development (NSHD, also known as 1946 birth cohort) study. It represents the oldest of the British cohort studies (620), and is unique in providing measures of LTL and CV risk factors at the ages of 53 and 60-64 years, together with a characterization of the cardiac and vascular phenotypes at the later time point.

7.2 Specific Methods

7.2.1 Population

The MRC NSHD is a social class stratified sample of all singleton births to married parents in England, Scotland, and Wales during 1 week in March 1946. The cohort originally included 2547 women and 2815 men and has been followed up 23 times since birth. The latest data collection took place when participants were 60-64 years old (between 2006 and 2010) (621).

Study members still alive and with a known current address in England, Scotland or Wales were invited for an assessment at one of six clinical research facilities or to be visited by a research nurse at home. Invitations were not sent to those who had died (n=778), who were living abroad (n=570), had previously withdrawn from the study (n=594) or had been lost to follow-up (n=564). Of those invited, 2229 (78%) were assessed: 1690 (59.2%) attended a CRF and 539 (18.9%) were visited at home (Figure 7.1).

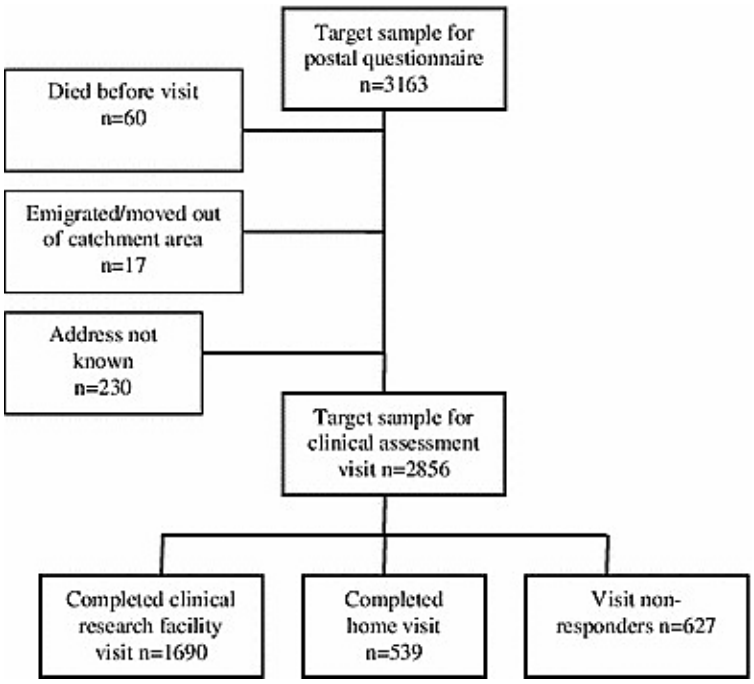


Figure 7.1 Response to invitations sent between 2006 and 2010 in the MRC NSHD cohort.
A total of 1690 patients attended the clinical facilities. This represented the target sample for vascular phenotype assessment.

The participating sample remains broadly representative of native born British men and women of the same age (622). At the age of 53 years, a team of trained nurses had visited survey members in their own homes (623).

At both 53 and 60-64 years, height, weight and waist and hip circumference were measured according to standardised protocols, two measures of systolic and diastolic blood pressure were taken with an OMROM 705 with the participant seated, with the second reading used in this analysis. Smoking status at both ages was collected by self-report questionnaire and individuals were categorised into current smokers and non-smokers. During home visits at 53 years and on postal questionnaires at 60-64 years, participants were asked to list all prescribed medications and to report doctor diagnosed myocardial infarction. Ethical approval

for the study was obtained from the Greater Manchester Local Research Ethics Committee and the Scotland A Research Ethics Committee for the 60-64 years collection and from the Multicentre Research Ethics Committee for the 53 years collection. Written, informed consent was obtained from the study members for each component of each data collection.

7.2.2 Biochemical tests

During the home visits at age 53 years, nurses took non fasting blood samples. Details of the biochemical assay used to estimate total cholesterol, HDL cholesterol and triglycerides have been previously provided (624). HbA1c was measured on a Tosoh A1c 2.2 analyser (625).

7.2.3 LTL assay

At both ages, DNA was extracted from frozen EDTA blood samples using Puregene DNA isolation kits (Flowgen, Leicestershire, UK). LTL at both ages was measured in the same laboratory according to a previously validated RT-PCR technique in a blinded fashion (71). Measurements were performed in quadruplicate on an Applied Biosystems 7900HT Fast Real Time PCR system with 384-well plate capacity. The intra-assay coefficient of variation was 2.7% while the inter-assay coefficient of variation was 5.1%. To correct for inter-plate variation, four internal control DNA samples of known telomere length were run within each plate and used to generate a regression line, by which values of relative telomere length for the actual samples were converted into absolute LTL in base pairs. LTL measurements were available in 2660 individuals at 53 years and 1058 individuals at 60-64 years. Using a conservative approach, we considered outliers and excluded from the analyses the 0.9% of participants who had a baseline LTL <2000bp or >11000bp.

7.2.4 Vascular Phenotype

The intima media thickness of the right and left carotid arteries were measured using the protocol described in the general methods. Briefly, the right and left carotid arteries were imaged longitudinally, one centimetre proximal to the carotid bifurcation following a standardized protocol (380). All measures were undertaken using an ultrasound scanner (Vivid I or Vivid 7, GE Healthcare) with a high resolution probe (12 MHz). Once clear images of the proximal and far walls IMT were obtained, the zoom function was used to magnify the region of interest. Ten second cineloops were recorded in DICOM format and downloaded for offline analysis. Analysis of the cineloops was performed in a core laboratory (Vascular Physiology Unit, Institute of Cardiovascular Science, University College, London) using dedicated software (Carotid Analyser, Iowa City, Iowa), which allows semi-automatic edge detection of the echogenic lines of the intima–media complex. Three end-diastolic frames were selected and analysed for mean cIMT of each image, defined as the interface between lumen-intima and media-adventitia. All images were analysed by two trained and blinded readers; intra- and inter-reader reproducibility were evaluated on a subset of 10 randomly selected images which were analysed in a blind fashion twice. Intra and inter-class correlations were >0.9. In our analyses, we included right (R_cIMT), left (L_cIMT) and the average between the two sides (cIMT).

7.2.5 Cardiac Phenotype

Participants underwent echocardiography performed by a trained, experienced sonographer using GE Vivid I machines. Echocardiographic images were obtained from parasternal long axis and short axis, apical 5-chamber, 4-chamber, 3-chamber, 2-

chamber and aortic views along with conventional and tissue Doppler in the 4-chamber view. Image analysis, including wall and chamber measurements for the evaluation of left ventricular mass, ejection fraction and diastolic function (estimated from the ratio between trans-mitral Doppler early filling velocity and tissue Doppler early diastolic mitral annular velocity, E/E′ as well as from early and late mitral inflow velocity, E/A) was undertaken in a single core laboratory according to ASE/EAE guidelines (626) by three experienced readers blinded to patient identity using GE EchoPac software (GE Connecticut, USA). Left ventricular mass (LVM) was indexed to body surface area (LVMI).

Quality assurance of echocardiography was performed throughout the study and blind duplicate reading reproducibility studies were carried out to establish inter- and intra-reader reliability. These showed excellent reproducibility (intra-class correlation coefficients were > 0.9).

7.2.6 Statistical analysis

Linear regression models were used to investigate the unadjusted association between LTL at 53 years, LTL at 60-64 years and their difference with each measure of vascular (cIMT) and cardiac (E/A, LVMI) phenotype at 60-64 years. A series of multiple regression models were then fitted to examine whether the traditional CV risk factors and other potential confounders influenced any associations observed between LTL and the vascular and cardiac phenotypes. LTL was standardised to have a mean of 0 and standard deviation of 1 for ease of interpretation of regression coefficients. First, adjustment was made for those variables which have been previously associated with LTL (age, gender, smoking and BMI) and second, additional adjustment was made for variables which, in our cohort, showed a

borderline association with cross-sectional measure of LTL or its change in linear regression models ($P<0.2$). In this regard we did not find significant associations between LTL and traditional CV risk factors (lipid profile, arterial hypertension or values of systolic/diastolic/pulse pressures, smoking, ethnicity, diagnosis of diabetes or levels of HbA1c and BMI) at 53 and 60-64 years. Only age and gender were significantly associated with LTL, while changes in BMI between the two visits presented a trend towards an association with LTL at 60-64 as well as its change between 53 and 60-64 year old. These associations however did not reach statistical significance. Accordantly, two multiple regression models are reported: model 1) adjusted for age at clinic visit (for 60-64 years analysis), gender, smoking, and difference in BMI (BMI at 60-64 minus BMI at 53); model 2) adjusted for relevant CV risk factors (cholesterol, systolic blood pressure), medications use, clinic centre, and past history of myocardial infarction. We report results with LTL shortening expressed as conditional change (the change is the regression coefficient for LTL at 60-64 years in a model including both LTL at 53 years and LTL at 60-64 years), unless otherwise specified. LTL change was also defined as absolute change (arithmetic difference in bp of LTL at 60-64 years minus LTL at 53 years), rate of change per year ([absolute change]/[difference in age between visits]), relative change ([LTL at 60-64 years - LTL at 53 years] / LTL at 53 years * 100) with consistent results.

In sensitivity analyses, we restricted our sample excluding first those using medication and then those without plaques. Lastly, participants were categorised into those with normal and those with increased cIMT values, using the threshold of the 75th percentiles of cIMT, as suggested by current guidelines and individuals with or without carotid plaque (14). The odds ratio for increased cIMT (binary variable > 75th

percentile) was calculated from multivariable logistic regression models with telomere length shortening both as continuous and categorical variables (>20%) and adjusting for the same confounders considered in the primary analyses. Analyses were performed using SPSS v21 (IBM, USA).

7.3 Results

7.3.1 Descriptive statistics

Characteristics of the study population at both time points are shown in table 7.1.

Table 7.1 Demographic, anthropometric and biochemical parameters of the MRC NSHD cohort

Variable	At 53 years (N=2611)	At 60-64 years (N=1207)
Gender, Male	1297 (49.7%)	--
BMI, Kg/m2	27.34±4.65	27.93±4.88
Systolic BP, mmHg	135.96±19.92	137.28±8.12
Diastolic BP, mmHg	84.34±12.06	78.36±9.83
HbA1C, %*	5.63±0.69	5.81±0.71
Cholesterol, mmol/l*	5.99±1.08	5.53±1.19
Triglycerides, mmol/*l	1.78±1.49	1.16±0.78
HDL, mmol/l*	1.59±0.48	1.54±0.41
cIMT		0.683 ±0.129
R-cIMT		0.666 ±0.134
L-cIMT		0.692 ±0.151
Eject. Fr. (%)		68.11 ±9.68
LA Dia (mm)		3.81 ±0.57
LVM/BSA (g/m^2)		92.46 ±27.12
E/A Ratio		0.97 ±0.27

*Values are presented as mean±standard deviation, *geometric mean±standard deviation or absolute number of participants and percentages (%).*
cIMT (Average carotid artery), R_cIMT (Right carotid artery), L_cIMT (Left carotid artery), LA Dia (LA Diameter) Eject. Fr. (Left Ventricle Ejection Fraction), LVM/BSA (Left Ventricular Mass/Body Surface Area), E/A Ratio (Early to Late Left Ventricular Filling Velocities)

The population was overweight with relatively high levels of systolic blood pressure, high levels of cholesterol and HbA1c. However, values of cIMT and cardiac parameters indicated healthy population, with normal left ventricular systolic and diastolic function, normal cIMT and preserved cardiac structure.

7.3.2 Associations between LTL at 53 and 60-64 years and CV phenotypes

LTL at 53 years was not associated with any vascular (cIMT) measures at 60-64 years (Figure 7.2).

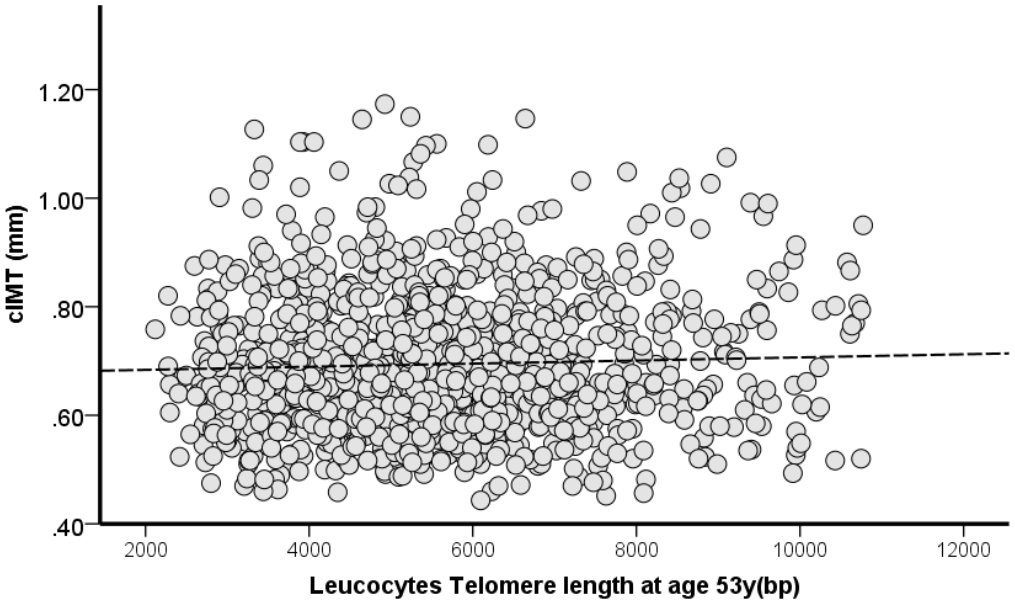


Figure 7.2 Association between LTL at 53 years and cIMT at 60-64 years.
LTL at 53 years did not predict the vascular phenotype assessed 10 years later

Similarly, there was no evidence of associations between LTL at 53 years and any of the cardiac phenotypes at 60-64 years old (Table 7.2).

Table 7.2 Mean difference (regression coefficient) of cardiac phenotypes against LTL at age 53 years

Variables		LTL
LA Dia	β *(95%CI)*	0.004 (-0.051, 0.059)
	P	0.887
Eject. Fr.	β *(95%CI)*	0.497 *(-0.367,1.360)*
	P	0.260
LVM/BSA	β *(95%CI)*	1.173 *(-1.625, 3.970)*
	P	0.411
E/A Ratio	β *(95%CI)*	0.004 *(-0.016, 0.024)*
	P	0.715

Linear regression model (unadjusted models)
cIMT (Average carotid artery), R_cIMT (Right carotid artery), L_cIMT (Left carotid artery), LA Dia (LA Diameter) Eject. Fr. (Ejection Fraction), LVM/BSA, E/A Ratio

At the age of 60-64 years, LTL was inversely associated with cIMT (regression coefficient (β)=-0.017mm per 1 standard deviation (SD) LTL, 95% confidence interval (CI): -0.031, -0.003 p=0.015) (Figure 7.3), although the association was greatly attenuated in the fully adjusted model (age, gender, smoking, body composition, cholesterol and systolic blood pressure at 60-64 years) (β=-0.008, 95%CI -0.023, 0.006 p=0.257).

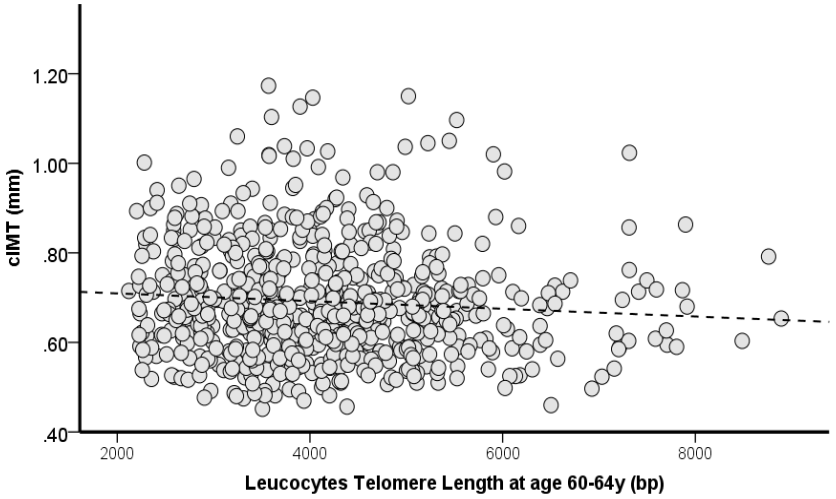


Figure 7.3 Cross-sectional association between LTL and cIMT at 60-64 years
A significant negative association was present between LTL and cIMT at 60-64 years. However, this association was greatly attenuated in the fully adjusted model.

No associations were observed between LTL at 60-64 years and cardiac measurements (table 7.3).

Table 7.3 Mean difference (regression coefficient) of cardio-vascular phenotypes against LTL at age 60-64 years

Variables		LTL
cIMT	β *(95%CI)*	-0.017 *(-0.031, -0.003)*
	p	**0.015**
R_cIMT	β *(95%CI)*	-0.018 *(-0.036, 0.001)*
	p	0.052
L_cIMT	β *(95%CI)*	-0.010 *(-0.021, 0.001)*
	p	0.071
LA Dia	β *(95%CI)*	-0.022 *(-0.071, 0.026)*
	p	0.364
Eject. Fr.	β *(95%CI)*	0.119 *(-0.676,0.914)*
	p	0.770
LVM/BSA	β *(95%CI)*	-1.987 *(-4.469, 0.494)*
	p	0.116
E/A Ratio	β *(95%CI)*	-0.001 *(-0.024, 0.021)*
	p	0.919

Linear regression model (unadjusted models)
cIMT (Average carotid artery), R_cIMT (Right carotid artery), L_cIMT (Left carotid artery) LA Dia (LA Diameter) Eject. Fr. (Ejection Fraction), LVM/BSA, E/A Ratio

7.3.3 Associations between longitudinal change of LTL and CV phenotypes

In 74% of subjects, there was telomere shortening over the follow up (Figure 7.4). A strong and inverse relationship was found between baseline LTL and rate of change in LTL during follow up ($r=-0.8$; $p<0.001$). Furthermore, men had a faster change of LTL during follow up compared to women ($p<0.01$).

A greater decrease in LTL between the ages of 53 and 60-64 years was associated with higher cIMT at 60-64 years old (β=-0.020mm per 1 SD decrease in LTL, 95%CI -0.027, -0.005 p=0.006) (Figure 7.4).

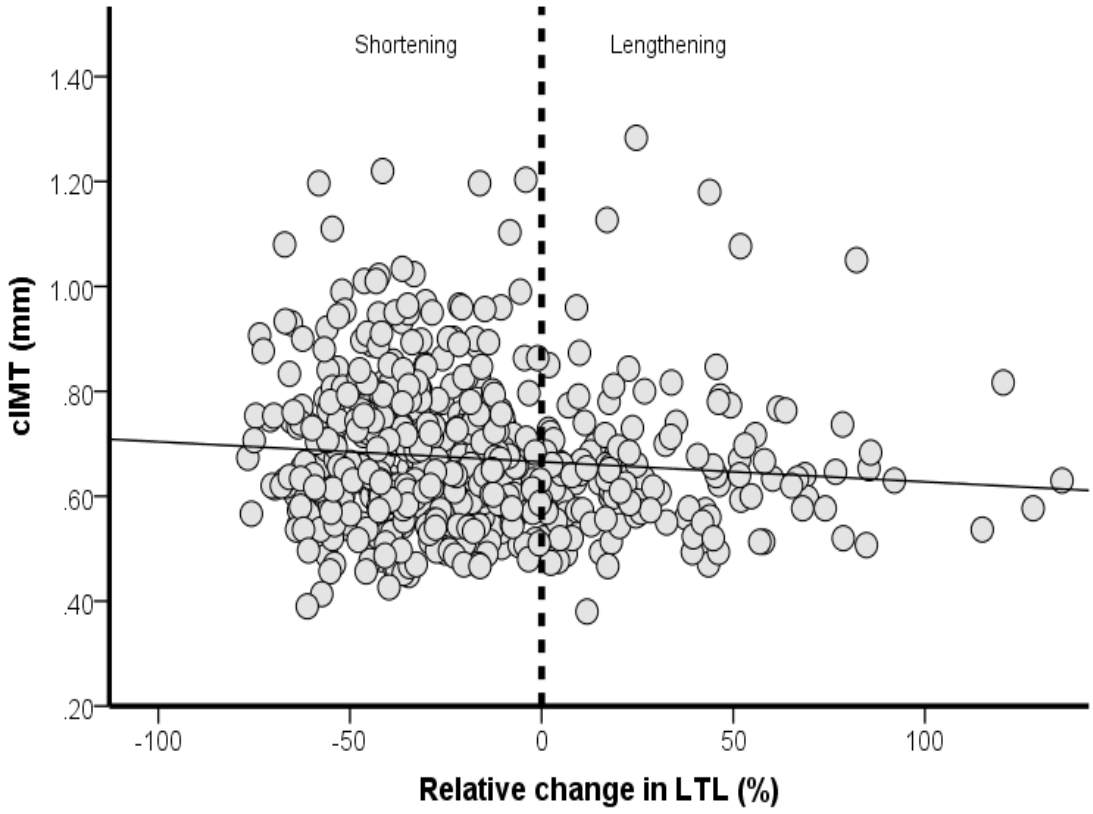


Figure 7.4 Association between percentage change LTL (LTL %) between 53and 60-64 years and cIMT at 60-64 years.
Subjects with faster rate of telomere attrition (or slower rate of telomere elongation) were those with higher final values of cIMT.

The strength of this association was not affected by multiple adjustment as described above (model 2: β=-0.015, 95%CI -0.026, -0.004; p=0.009) (table 7.4). The right cIMT showed very similar findings, while a weaker association was found with the left cIMT, which became non-significant after adjustment (Table 7.4).

Table 7.4 Mean difference (regression coefficient) of cIMT per 1 SD higher change in LTL (change conditional to baseline LTL).

(N=672)	cIMT (mm)		R_cIMT (mm)		L_cIMT (mm)	
	β *(95%CI)*	p	β *(95%CI)*	p	β *(95%CI)*	p
Unadjusted	-0.020 *(-0.027, -0.005)*	**0.006**	-0.019 *(-0.032, -0.006)*	**0.004**	-0.014 *(-0.027, -0.001)*	**0.033**
Model 1	-0.015 *(-0.026, -0.003)*	**0.011**	-0.019 *(-0.032, -0.006)*	**0.005**	-0.012 *(-0.025, 0.001)*	0.068
Model 2	-0.015 *(-0.026, -0.004)*	**0.009**	-0.019 *(-0.031, -0.006)*	**0.003**	-0.013 *(-0.029, 0.002)*	0.095

Generalized linear model
Model 1= Age difference in years, Gender, Smoking Status 99, Difference in BMI (99-09),
Model 2= Model 1+ Cholesterol 2009, Systolic BP 2009, Anti-inflammatory use, OAC, Lipid Lowering, Clinic Centre and History of MI
cIMT (Average carotid artery), R_cIMT (Right carotid artery), L_cIMT (Left carotid artery).

No associations were found between changes in LTL and cardiac phenotype measures of left ventricular mass, ejection fraction and diastolic function (Table 7.5).

Table 7.5 Mean difference (regression coefficient) of cardiac phenotypes per 1 SD higher change in LTL (change conditional to baseline LTL).

	(N=672)	Unadjusted
LA Dia (mm)	β (95%CI)	0.006 *(-0.004, 0.015)*
	p	0.239
Eject. Fr. (%)	β (95%CI)	-0.004 *(-0.013, 0.005)*
	p	0.423
LVM/BSA (g/m^2)	β (95%CI)	-0.006 *(-0.024, 0.012)*
	p	0.501
E/A Ratio	β (95%CI)	-0.001 *(-0.020, 0.017)*
	p	0.881
E/E' Ratio	β (95%CI)	0.009 *(-0.011, 0.029)*
	p	0.376
E'/A' Ratio	β (95%CI)	0.007 *(-0.015, 0.028)*
	p	0.544
E'	β (95%CI)	0.014 *(-0.001, 0.029)*
	p	0.067

LA Dia (Left Atrial Diameter), Eject. Fr. (Left Ventricular Ejection Fraction), LVM/BSA (Left Ventricular Mass indexed to Body Surface Area), E/A Ratio (Early to Late Left Ventricular Filling Velocities), E/E' Ratio (ratio between trans-mitral Doppler early filling velocity and tissue Doppler early diastolic mitral annular velocity).

Similarly, a greater shortening of LTL was associated with higher odds of increased cIMT (OR 1.97 [1.18-3.29], P= 0.01 for LTL shortening as continuous

variable and OR 1.79 [1.19-2.68], P<0.01 for LTL shortening > 20%). Individuals with carotid plaques, had higher shortening of LTL during follow up compared to those without plaques (P<0.01) . Sensitivity analysis did not affect the results.

7.4 Discussion

This study explored for the first time the association between changes in LTL over 10 years and CV phenotypes. We found, using data from a nationally representative cohort of 60-64 year old men and women, that a faster rate of LTL attrition over 10 years was associated with increased cIMT. This association was slightly weaker for the left compared with the right cIMT, was seen in participants with both elongation and shortening of LTL and persisted after adjustment for potential confounders and mediators. Cross-sectional and longitudinal variations of LTL were not related with cardiac phenotypes. These results suggest that, over and above the contribution of classic CV risk factor exposure, part of the variation in vascular phenotype in late midlife is influenced by mechanisms regulating the rate of cellular aging.

Several studies have reported cross-sectional associations between shorter LTL with subclinical and clinical markers of atherosclerosis in middle age populations (353;354;543). The lack of longitudinal data on telomere trajectory, however, limits the degree to which causal inferences can be made. This study showed that the negative relationship between LTL and cIMT described at 60-64 year old is likely to be dependent upon a faster rate of LTL change during follow up, suggesting that regulation of cellular aging (reflected by LTL dynamics) has a role in the process of vascular remodelling. A recent sub-analysis of the Cardiovascular Health Study

supports this hypothesis, demonstrating that single nucleotide polymorphisms in genes which regulate LTL dynamic are able to predict CV mortality (627).

An explanation for the association between short LTL and higher levels of vascular damage lies in the unique ability of LTL to provide information on the balance between lifelong exposure to injurious factors and the ability to repair within the CV system (240). Oxidative stress exposure is currently considered the main driver of atherosclerosis (628;629). Similarly, an elevated burden of oxidative stress can cause a faster rate of LTL attrition by increasing the oxidative stress-mediated damage to the telomere sequence (33;42;43). Therefore, exposure to increased levels of oxidative stress may explain our findings. On the other hand, due to the hierarchical organization of the hematopoietic system, LTL dynamics mirror at any age telomere dynamics in hematopoietic stem cells (HSCs), which also represent the haematological precursors of endothelial progenitor cells (113). Therefore, a faster rate of LTL shortening may reflect a faster rate of telomere attrition in HSC reserves, ultimately resulting in a limited ability of the bone marrow to supply an adequate number of endothelial progenitor cells for effective repair of vascular damage (240).

The lack of relationship of CV risk factors with LTL and its dynamic change is intriguing as it supports the hypothesis that, over and above the contribution of CV risk factor burden, a proportion of cIMT variability in our cohort depends exclusively upon the physiological evolution of cellular aging processes. Several studies have reported cross-sectional associations between LTL and smoking, obesity and levels of insulin resistance (66;67). Consequently, it has been suggested that an increased CV risk factor burden could represent the primary mediator of the association between LTL and vascular phenotype, leading to a faster rate of LTL shortening and vascular

remodelling (541). Although this hypothesis cannot be excluded, the few investigations that have analysed the determinants of LTL shortening in healthy and diseased populations found limited or no influence of CV risk factor levels on the rate of LTL attrition (87;116;117). This evidence raised doubts on the ability of traditional CV risk factors to influence effectively LTL dynamics and to mediate its association with CV phenotypes. This study demonstrates that the association between LTL dynamics and markers of CV disease is unlikely to be dependent on traditional CV risk factor exposure, suggesting that other factors such as oxidative stress or genetic mechanisms which regulate the progression of cellular aging could drive this association.

Inconsistent associations have been reported between LTL and cardiac phenotypes. In a cross-sectional analysis in the Framingham Heart study, Vasan et al found a positive association between LTL and LVM (630). In contrast, the Newcastle 85+ study reported no cross-sectional association between LVM and LTL, despite demonstrating a positive relationship between LTL and left ventricular ejection fraction (631). Finally, in a small cohort of 334 participants, Kuznetsova et al reported that the baseline measure of LTL predicted LVM over 7.4 years of follow up (632). In our study, there was no association between cross-sectional and longitudinal measures of LTL and cardiac phenotype, in line with current understanding of the LTL biology and its relevance to CV disease. Given that the hematopoietic system is the most proliferative among human tissues, age-dependent telomere shortening in HSCs, as expressed in LTL dynamics, is unlikely to reflect the evolution of the aging process of cells with slow turnover rate, such as cardiomyocytes. Our results supported this view and provide the first epidemiological evidence of a possible different role of LTL biology in the evolution of cardiac and vascular remodelling.

Individuals with longer LTL at baseline had a faster rate of LTL attrition during follow up. Obviously, this finding could result from mathematical coupling of the baseline and follow-up measure of LTL (see the paragraph "Unexpected findings from longitudinal measurements of LTL" in the introduction). However, similar results had previously been observed in the Bogalusa Heart study and in the Heart and Soul Study (87;116;117). Two possible explanations have been provided for this finding. The first allocates a crucial role to post-natal environmental exposure, suggesting that longer telomeric regions may be at increased risk of oxidative stress damage due to their higher content of guanine bases (124;125). The second hypothesis is focused on the regulation of LTL by telomerase activity. Indeed, longer telomeres are more likely to acquire a T-loop conformation, which precludes access of telomerase to the telomeric region (123). Subsequently, shorter telomeres become privileged targets for telomerase activity and are more likely to undergo elongation than longer telomere sequences.

While linear regression analyses showed that the influence of LTL dynamics on cIMT was small, it was remarkable that one standard deviation change of LTL almost doubled the odds of greater cIMT at 60-64 years old. Brouilette et al. reported similar odds when they related baseline measures of LTL with future CV risk (61), suggesting the measure of LTL dynamics could represent a novel tool that might help identify individuals in the general population at increased risk of CV disease beyond traditional risk factors.

Finally, females had a lower rate of LTL compared to males. While these results were in contrast with previous findings (62;87;95;543;630), they confirmed the lack of an influence of the oestrogen exposure on the LTL dynamics. If the anti-

inflammatory and anti-oxidative effects of oestrogens were involved in the regulation of LTL dynamics, one would have expected that, at the end of the reproductive age (52 years), females had longer LTL than males. Similarly, the lack of oestrogen exposure between 52 and 60-64 years, should have led to a similar rate of LTL shortening during follow up. These assumptions were not confirmed in this study, as women had shorter LTL at baseline and tended to maintain the measure of LTL during follow up. This confirmed the hypothesis that genetic, rather than post-natal factors may regulate the gender differences in LTL dynamics. As mentioned in our third study, several observations suggest that gene variance on the X chromosome exerts an important influence on telomere length dynamics (97).

The MRC NSHD has a number of strengths for the investigation of LTL biology and its relationship with CV risk factors and phenotypes. Firstly, the NSHD study is the only cohort in which longitudinal measures of LTL have been made together with a comprehensive characterization of a range of cardiac and vascular phenotypes. Secondly, the 10 year follow up between LTL measures and the relatively large number of participants included in the analysis, increases the power of the results. The yearly rate of change of LTL is low when compared to the ability of current LTL assays to detect small differences in telomere length. Consequently, changes in LTL over a long follow up period and a large sample size are necessary to detect inter-individual variations in telomere attrition rates with confidence. Furthermore, the availability of data on a wide range of established and novel CV risk factors and potential confounders at both ages allowed, for the first time, investigation of the independent contribution of LTL dynamics to CV phenotype.

This study has limitations. Firstly, the reported associations are observational, and therefore no definitive conclusions can be made regarding causality. Secondly, although we adjusted for multiple potential confounding variables, the possibility of residual confounding cannot be excluded. For example, we did not adjust for levels of inflammatory markers, oxidant and antioxidant molecules (e.g. homocysteine, vitamin D, omega-3 fatty acids, etc.), factors that have been previously shown to influence cross-sectional and longitudinal measures of LTL (117;243;251). Thirdly, stored whole blood samples were used to measure LTL. This allowed us to document a relationship between LTL dynamics and CVD but it is impossible to test whether the same relationship holds for other age-related diseases. Leukocytes are inflammatory cells which are exposed to the same inflammatory environment of the vascular wall. Therefore, the link between CVD and LTL could arise from the common influence of CV inflammation on both LTL dynamics and atherosclerosis (75;240). However, several reports documented a high synchronization between LTL and telomere length of other tissues, suggesting that LTL could be used as a reliable marker of whole body rather than CV aging and inflammatory burden (88;89;516). Finally, the qPCR-based LTL assay measures the average telomere length across all leukocytes in the peripheral blood. We were therefore unable to compare telomere length and their rate of shortening between different leukocytes subpopulations. Nevertheless, several reports have demonstrated that the common origin of leukocytes from the HSCs makes their telomere length highly synchronized at any age (113;515). Larger longitudinal studies with measure of LTL at multiple time points are required to confirm our findings.

In conclusion, our results suggested that biological pathways regulating the process of cellular aging set a higher risk of vascular damage in adulthood. This

process of “vascular aging” was only partially affected by exposure to CV risk factors. Factors involved in the post-natal regulation of LTL are likely to play a central role in the initiation and evolution of atherosclerosis and may help in identifying individuals in the general population with higher susceptibility to CV disease.

KEY POINTS

Faster rate of telomere attrition relates with increased cIMT, suggesting a role of biological aging pathway in the progression of vascular disease

The proportion of cIMT explained by the rate of LTL attrition is independent from cardiovascular risk factors, suggesting that, over and above the influence of the cardiovascular risk burden, there might be a process of vascular aging which contributes to progression of CVD

LTL is unlikely to influence cardiac phenotypes, probably because of the low rate of replication of myocardiocytes

8 FINAL DISCUSSION

This thesis aimed to address the impact of one proposed driver of senescence, the cumulative exposure to inflammation and oxidative stress, on a specific cellular aging pathway, telomere length, in humans. The key finding is that long term inflammatory burden is the most likely mediator of the association between LTL and age related diseases. In adulthood, levels of inflammatory markers are unable to inform on the cumulative impact of inflammation on LTL and age-related diseases. Only clinical markers reflecting the long term exposure to inflammation are strongly associated with LTL. Conversely, in young subjects with limited inflammatory burden, circulating inflammatory markers may help in identifying people with the higher risk of short LTL. Finally, the association between rate of telomere shortening and cIMT suggests that the relationship between short LTL and age-related disease, which have been reported in several adult studies, is likely to dependent upon post-natal mechanisms regulating the LTL dynamics.

These findings suggest that chronic exposure to low levels of inflammation (such as those encountered in normal life) can lead to the evolution of a physiological aging process which occurs even in the absence of overt disease and starts very early in life. This process can be cranked up by increased levels of inflammatory and oxidative stress exposure, leading to emergence of diseases commonly detected in the elderly (i.e. CVD). The ability of LTL to record the chronic burden of inflammation makes this measure a reliable aging marker as well as a possible predictor of age-related diseases. It remains unclear whether the biological processes leading to healthy or unhealthy aging can be effectively counteracted by reducing inflammation. However, this thesis suggests that potential strategies aimed to reduce inflammatory burden to prevent evolution of aging should start from the earliest phase of life.

Using two human models of chronic inflammation (chronic PD and atherosclerosis), this PhD highlighted the limitations of common inflammatory markers (IL-6 and CRP) in informing on the individual's cumulative inflammatory burden. Chronic PD induces a mild but chronic state of systemic inflammation which has been repeatedly associated with an increased risk of age-associated diseases including cancer, atherosclerosis, insulin resistance/diabetes, osteoporosis, decline in renal, lung and cognitive functions (299-320). Importantly, while these studies repeatedly documented a continuous relationship with markers of oral disease severity, none reported an association between circulating markers of inflammation and risk of age-related diseases. This is expected, as CRP and IL-6 are acute phase reactants and their levels in subjects with an underlying inflammatory disease are more likely to reflect the inflammatory state at the moment of blood collection, rather than informing on long term inflammatory burden (254-262). In contrast, the structural and functional modifications induced in different tissues by the inflammatory burden are more likely to reflect long term rather than acute inflammation. For example, in patients with PD, the measures of CAL is likely to provide more accurate information on the cumulative inflammatory burden than a single measure of CRP and IL-6 (501;524;633). CAL is a clinical approximation of the loss of connective tissue attachment from the root surface and provides an objective site-by-site assessment of the long term amount of periodontal damage and inflammation (501). When bacteria accumulate on the teeth, the host mounts an inflammatory response in the approximating gingival tissue. Such acute inflammatory response can be beneficial because in the short term, as it a) prevents bacterial growth in the tissue, b) removes bacterial products such as antigens, LPS, and enzymes that have penetrated the tissue and c) is associated with specific antibody formation that,

in some cases, appears to be protective (634;635). However, if the inflammatory trigger is not efficiently removed, the persistent perturbation of the local tissue homeostasis leads to the definition of new pathological set points, in an attempt to deal better with the extreme condition (636-641).

Atherosclerosis represents another human model of chronic inflammation which can be used to study the complex relationship between inflammatory burden, markers of inflammation and age-related disease. While atherosclerosis is recognised as a chronic inflammatory state of the vascular wall, the ability of CRP, fibrinogen and IL-6 to predict the risk of CV morbidity and mortality remains highly controversial (642-646). Several studies have demonstrated that the addition of CRP to the Framingham risk score does not provide substantial reclassification improvements of patients at intermediate to high risk for CV disease (647-657). Conversely, measures of vascular damage (such as cIMT and coronary artery calcium), which integrate the long term variable inflammatory burden of each individual, add important prognostic information to the common scoring systems used to estimate CV risk (361;647;658). While the inflammatory trigger is different, the processes which lead to the accumulation of inflammatory cells in the vascular wall of patients with atherosclerosis are similar to those described in patient with chronic PD (173;332;659;660). The chronic inflammatory process induces structural changes of the vascular wall reflected by progressive increased thickness of the intima media layer and a progressive reduction of vascular elasticity (607;608). Therefore, as CAL provides a reliable marker of chronic gingival inflammation, as well cIMT provides a reliable marker of the chronic vascular inflammation and oxidative stress burden in each individual.

The ability of inflammation to affect LTL relates to the two major factors which have been shown to impact on LTL dynamic: the end-replication problem and oxidative stress (33;40). When monocytes/macrophages fail to remove the bacterial biofilm accumulating on the teeth or oxLDL molecules from the intima layer, they shift their pattern of cytokine production to increase the recruitment of novel inflammatory cells and to limit the virulence of the inflammatory trigger (40;661;662). The highlighted recruitment of inflammatory cells in the gingival or vascular inflammatory processes is achieved with the cost of an increased proliferation of the bone marrow HSCs, which represent the haematological precursors of most of the inflammatory cells circulating in peripheral blood (40;663). This increased rate of cell replication and differentiation cause faster telomere attrition in HSCs, due to the end replication problem. While HSCs have a residual telomerase activity, the new homeostatic set point defined by gingival or vascular inflammation is likely to overcome the ability of telomerase to replace the telomere sequences loss during cells replication (664). As a consequence, subjects with longer history of inflammatory burden are likely to represent those with shorter LTL. In this context, the increased levels of systemic oxidative stress might further enhance the rate of telomere shortening in HSCs. As per its impact on cultured cells (42;43), oxidative stress probably augments telomere shortening by two major mechanisms:

- Inducing single and double strand DNA breaks which increase the telomere loss in HSCs per cell replication
- Diminishing the biological life of a subset of leukocytes which replicate in the peripheral circulation (i.e. lymphocytes), further enhancing HSC replication to accommodate peripheral needs.

The hypothesis a lifelong rather than acute exposure to inflammation is the main driver of LTL shortening is further supported by the third study, which suggests that the adult association between LTL and markers of chronic inflammation is likely to represent a late reflection of biological pathways acting from younger ages. While the association between markers of inflammation (CRP and fibrinogen) and LTL described in the Ten Towns cohort is apparently in contrast with the first two studies, it is important to remark that the environmental burden is limited in adolescence, making levels of common inflammatory markers more likely to reflect the baseline inflammatory burden rather than the presence of subclinical underline diseases. Concordantly, the concentrations of CRP and fibrinogen reported in this young cohort are well below those recorded during acute or chronic inflammatory responses/diseases (665). The detailed cohort characterization undertaken in this study has allowed clearly demonstration that the impact of inflammation on LTL is independent from CV risk factors, behavioural and socio-economic factors, previously associated with LTL (67;581;594-597). This suggests that the link between LTL and inflammation anticipates and possibly explains the adult associations between LTL and patterns of dyslipidaemia, blood pressure, obesity and insulin resistance.

Patient with diabetes who develop PD have shorter LTL compared to those without the oral disease. This evidence suggests that the relationship between clinical markers of chronic inflammation and LTL is graded and continuous, without obvious thresholds. This is an important finding as it confirms that the oxidative stress damage plays a crucial role in regulating the rate of telomere attrition. Indeed, previous studies have demonstrated that patient with diabetes have shorter LTL compared to the general population (62;63;66;250;518). Cells with significantly short telomere length have a reduce replication capacity (28). Therefore, in patients with baseline short LTL

(i.e. diabetic subjects), the development of a second inflammatory disease (PD) cannot be associated with a significant increase in the rate of cell replication. Indeed, several report documented that the number of progenitor cells derived from HSCs is decreased in the peripheral circulation of both type 1 and type 2 diabetes and that this alteration is likely to be involved in the pathogenesis of diabetic complications (666-668). Consequently, the shortened LTL recorded in patient with diabetes and PD compared to those with diabetes alone is likely to depend more on the oxidative stress telomere damage than on the end-replication problem. On the other hand, the shortened LTL recorded in diabetic patient with severe PD can compromise the ability of the immune system to recruit new inflammatory cells to properly counteract the aggression of the oral bacteria. Indeed, with a histological analysis of 22 biopsies of untreated PD Chapple at al. demonstrated an apparent failure of recruitment and activation of macrophages when compared with 26 biopsies of clinically healthy (minimally inflamed) gingival tissue (669). Therefore, the continuous relationship between BEP and LTL observed in the second study may underscore an increased rate of progression of the periodontal disease resulting from a deficient activity of the immune-inflammatory system.

All this evidence suggests that LTL may represent a new reliable marker to record the individual's burden of inflammation and oxidative stress across lifespan. Furthermore, because inflammation and oxidative stress are at the centre of hypotheses on the development and progress of the aging process, the unique feature of LTL as a record of the cumulative burden of inflammation and oxidative stress might explain its association with aging-related diseases.

Over and above the influence of inflammation, only gender has an independent impact on LTL. In all studies presented in this thesis as well as in most of those reported in the literature (62;88;95;543;630), females have longer LTL than males. The fourth study suggest that this is likely due to a faster rate of LTL attrition in males compared to females, a condition that also predispose to an increased risk of age related disease, such as atherosclerosis. In the developed world in modern times, life expectancy at birth is some 7 years longer for women than it is for men (670), indicating that women age more successfully than man. Reasons which account for this longevity gender gap remain unknown, but LTL could provide a possible biological pathway accounting for this difference. Indeed, the longer LTL recorded in women could protect them from developing age-related diseases and, consequently, it could provide them with a survival advantage compared to men. While from this thesis it is impossible to identify clear mechanisms leading to longer LTL in females than males, several hypotheses can be excluded.

The predominant thinking has been that centrally controlled processes mediated by ovarian steroid hormones--notably estrogen--underlie the longevity gender gap (671). Estrogen and its derivatives exert potent antioxidant effects via a number of mechanisms, including scavenging free radicals, inhibiting free radical formation, and stimulating enzymes which are crucial for free radical detoxification (672). The reduced bioavailability of free radicals could protect women from developing age related diseases, including atherosclerosis and cancer and, at the same time, it could also reduce the level of LTL oxidative stress damage. This anti-oxidant effect observed in women together with the ability of estrogen to stimulate the transcription of the gene encoding the telomerase reverse transcriptase enzyme, can curtail or slow down the rate of telomere erosion (672-674). Although this hormonal hypothesis can

explain the longer LTL in female compare to males, there are at least three findings in this thesis which suggest that alternative factors may be involved in defining the LTL gender gap:

1. A significant sex difference in LTL of 138bp is detectable already at 13-16 year old, when the duration of the estrogen exposure is limited to only 2-3 years. If only 3 years of estrogen exposure can determine a gender gap of 138bp in LTL, this should correspond to a net gain of 40-70bp telomeric repetitions per year of estrogen exposure. Assuming that the impact of estrogen on LTL is constant throughout reproductive lifespan and that the duration of the reproductive age is 35-40 years, this difference will lead to a final gender difference in LTL ranging from 1400 to 2800 bp, which is significantly higher than that normally recorded in other adult studies (62;87;95;543).
2. There was no association between grade of pubertal maturation and LTL as well as no gender interaction was found in the association between LTL and inflammatory markers. This further supports the hypothesis that duration of estrogen exposure does not influence LTL by attenuating the impact of inflammation.
3. The rate of LTL attrition between the ages of 53 to 60-64 years remains higher in males compared to females. As at 53 year old most females are likely to be in menopause, the rate of LTL should be equal between genders, due to the lack of the antioxidant effects of estrogen.

In addition to the possible role for estrogens, two more possible explanations for the gender gap in LTL could be conceived. Firstly, several lines of evidence suggest a X-linked inheritance and regulation of LTL (96;97;101). In patient with X-linked

dyskeratosis congenita, for example, the substitution of an amino acid in dyskerin (a protein that modifies ribosomal RNA and binds telomerase), leads to shortened telomere length, bone marrow failure, and thus premature demise in affected men (675). It is therefore likely that a higher level of expression of genes located on the X chromosome in females compared to males is responsible for variation in telomere length between genders. The presence of an X linked inheritance and regulation LTL dynamic is partially confirmed by a report of Nawrot et al., who described robust correlations in adult LTL between fathers and daughters, mothers and sons and daughters, and among siblings (97). Secondly, in normal females, one of the X chromosomes is stochastically inactivated during early embryogenesis (676), so that no more than ~25% of genes on the inactive X chromosome are expressed (677). Newborn girls therefore have two populations of somatic cells at an approximate ratio of 50:50, exhibiting balanced mosaicism with respect to X inactivation. With advancing age, the repeated HSC replications might lead to selection of those cells with an activated X chromosome harbouring the genetic code that causes longer telomeres. This occurrence was reported in carriers of X-linked dyskeratosis congenital (59). TRF length of the inactive X chromosome is closely similar to that of the active X chromosome in normal newborn girls, but is shorter than the active X chromosome in older women (678).

The relationship between LTL and vascular damage is more complex than that observed for other age-related diseases. By virtue of its anatomy, the vascular endothelium interacts with elements on its luminal and counter-luminal sides, playing key roles not only in the biology of the vasculature but also in that of the blood. Importantly, the vascular endothelium is where atherosclerosis evolves (324). Based

on a few autopsy cases, it was proposed that telomere shortening in the endothelium and consequently endothelial cell senescence contribute to the atherosclerotic process in the coronary arteries (350). Given the relatively low replicative index of endothelial cells (and smooth muscle cells) in the vascular wall, it is unlikely that these cells would experience senescence from telomere shortening during the human lifespan. If telomere dynamics are involved in atherosclerosis, their main impact might be not on native endothelial cells in the vascular wall but on EPCs. These cells are engaged in maintaining the integrity of the vascular endothelium (679), but their number and proliferative potential, relate to their telomere length (680-682). Importantly, as both EPCs and leukocytes originate from the same haematopoietic precursor (HSCs), the telomere length dynamics in EPCs pool are mirrored by LTL dynamics (113;515). Subsequently, by measuring telomere length of peripheral leukocytes it is possible to obtain information on the residual repair capacity of EPCs. The shortened LTL recorded in individuals with atherosclerosis not only forecasts an increased oxidative stress and inflammatory burden, but also marks a reduced ability to repair the vascular damage. Indeed, in individuals with atherosclerosis it is common to observe short LTL (61;63) and a reduced number and proliferative potential of EPCs (683-687).

While there is several evidence in support of a strong synchronization of telomere length in leukocytes and EPCs (113;515), it remains unknown whether the increased risk for atherosclerosis expressed in the form of shortened LTL during adulthood results from short telomere length at birth or their accelerated attrition rate afterward. Biologically, both these circumstances can confer an increased risk of atherosclerosis. In individuals with short LTL/EPC telomere length at birth, the increased risk of atherosclerosis is likely to depend on a reduced ability to repair the vascular damage (680-682) rather than increased levels of post-natal environmental exposure. On the

other hand, individuals with faster rate of LTL attrition may represent those with normal ability to repair but increased exposure or susceptibility to inflammation or oxidative stress in post-natal life (75;241). In this thesis, it was not possible to report measure of LTL at birth with a long term follow up, but the results obtained in the 1946 Birth Cohort are in agreement with the second rather than the first hypothesis. Indeed, LTL at baseline (53 year old) did not predict the amount of vascular remodelling recorded 10 years later, suggesting that a primary deficit of the ability to repair is unlikely to significantly increase the risk of CVD. Conversely, the rate of LTL shortening between the two visits was associated with the burden of vascular damage and led to the expected inverse association between LTL and cIMT observed at the age of 63 years. These results suggest that post- rather than pre-natal factors are the main determinants of the risk of short LTL and atherosclerosis in adulthood. This is extremely relevant in the management of patient at risk for CVD. As the rate of telomere length attrition, rather than their size at birth, is the main determinant of the association between short LTL and CV risk in adulthood, an aggressive treatment of all factors increasing the inflammatory burden in post-natal life is likely to reduce the risk of CVD, slowing the progression of cellular aging.

The results obtained from the 1946 Birth Cohort Study highlight a second important feature of LTL biology. While LTL dynamics reliably mark the evolution of the aging phenotype in highly proliferating tissues, they do not provide similar information for tissues with low replicative potential. Indeed, the rate of change of LTL is strongly associated with the levels of vascular wall remodelling, but does not predict the evolution of cardiac damage. This is expected as endothelial cells lining the inner surfaces of the vessels have a turn-over significantly higher than myocardiocytes. The hypothesis that LTL dynamics are poor markers of disease

evolution for tissues with low replicative activity, such as skeletal muscle or neurons, is confirmed by several reports. For example, telomere length biology does not explain the evolution and severity of diseases characterised by degeneration of skeletal muscle fibres, such as Duchenne muscular dystrophy (688). Further, while LTL is reduced in patient with stable mild cognitive impairment (689;690), telomere length does not predict conversion to Alzheimer's disease (690). Short LTL however is associated with higher risk of idiopathic pulmonary fibrosis and cryptogenic liver cirrhosis, diseases characterised by altered function and renewal of the highly proliferating pulmonary alveolar cells and hepatocytes, respectively (691;692). This evidence, together with the findings of this thesis, suggests that mechanisms regulating telomere dynamics in peripheral leukocytes are likely to involve also other highly proliferative tissues, making LTL a reliable marker to follow the evolution of the aging phenotype in tissues with high replicative potential. This will explain the associations between LTL and age-related diseases involving also tissues external to the haematological compartment.

8.1 Limitations

Most studies report cross-sectional associations between LTL and inflammatory markers and, therefore, may only suggest causality. It is impossible to define whether short telomeres are cause or result of an increased exposure to inflammation. This however does not reduce the relevance of current findings. Indeed, if short telomeres lead to a higher inflammation, this could explain the increased levels of inflammatory markers recorded in the elderly and, at the same time, their higher predisposition to age-related diseases. Secondly, the human population studies performed in this thesis

only rely on the mean telomere length of blood cells. Although these studies have uncovered the association between lower values of mean telomere length, increased inflammation and greater risk to develop several age-related diseases, recent reports have documented the importance of measuring the frequency of short telomeres, rather than the mean telomere length, as indicator of telomere dysfunction (693;694) and, subsequently, of cell and tissue dysfunction. Indeed, critically short telomeres cannot be repaired by any of the known DNA repair mechanisms and consequently trigger a persistent DNA damage response (DDR), which leads to cellular senescence and/or apoptosis (53;54), eventually compromising tissue regenerative capacity and function, and contributing to organismal aging (40). Thirdly, the relationship between markers of tissue remodelling and LTL has been interpreted as an early exhaustion of the regenerative ability of the local tissues which would be reflected by short telomere length in peripheral leukocytes. Nevertheless, given the lack of data regarding the changes in telomere length from vascular and gingival tissues, this can be only considered one hypothesis. Finally, the possible impact of oxidative stress on the telomere sequence has not been explored directly. No measures of telomere oxidation or telomere oxidative stress damage have been reported.

8.2 Clinical relevance of the study

The key finding of this thesis is that chronic but not acute exposure to inflammation and oxidative stress is likely to impact on cellular aging pathways in humans. While chronic exposure to inflammation has long been considered the driver of human aging (4;225;695), the lack of markers which could inform on the

cumulative inflammatory burden has strongly limited the ability to prove this hypothesis at a population level. In large epidemiological studies, the addition of CRP, IL-6, fibrinogen, etc. to scoring systems used to classify individuals at low, intermediate and high risk for age-related did not provide a significant reclassification improvement (647-657;696;697). When markers of cumulative inflammatory burden are added to scoring systems, they are likely to improve the ability to identify those individuals at risk to develop age-related disease in the general population. One example is provided by CAD. The addition to the Framingham risk score of parameters reflecting the cumulative levels of vascular damage related to inflammation (such as cIMT or coronary artery calcium), has shown to improve our ability to identify subjects with increased risk of CV morbidity and mortality in the future (361;647;658).

The key role of chronic inflammation in the evolution of aging and age-related diseases in humans is confirmed by the ability of anti-inflammatory drugs (i.e. aspirin) to reduce the risk of several age related diseases, such as cancer and CVD. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have received considerable interest as potential cancer chemopreventive agents due to their anti-inflammatory properties (698-700). Animal studies suggest that these agents may inhibit tumor growth by modulating cellular proliferation via suppression of endogenous prostaglandin synthesis through inhibition of cyclooxygenase (COX) enzymes (701;702). Aspirin is also considered to be a chemopreventive agent because of its antioxidant properties (703;704). Observational studies (705-722) and randomized trials (723-729) have evaluated the effect of aspirin and non-aspirin NSAIDs on site-specific cancer incidence, particularly in the colon (730), breast (731), pancreas (732), blood (leukemia, (733)) and, more recently, skin. They have

demonstrated chemopreventive benefits from aspirin and/or non-aspirin NSAIDs on risk of cancer. Three recent studies by Rothwell and colleagues confirmed the strong chemoprotective effect of aspirin (734-736), also showing that the drug is effective in reducing the risk of distal metastasis (737). The beneficial effects of aspirin have also been detected in CAD prevention. The cardioprotective effects of aspirin are mostly related to its anti-platelet activity, which prevent thrombus formation on vulnerable plaques. However, the reduction of CVD risk associated with the use of aspirin appears to be directly related to the reduction of the C-reactive protein levels, suggesting that anti-inflammatory properties of the drug may have clinical benefits in preventing CVD (738).

These reports support chronic inflammation as potential target for a more aggressive prevention of age-related frailty and mortality in the general population. However, the possible biological pathways explaining the associations between inflammation and age-related disease remained unknown. This thesis identifies LTL and its dynamic regulation as potential biological mechanisms accounting for associations between age-related disease and inflammatory/oxidative stress exposure. Remarkably, results of the Ten Towns study suggests that the inflammatory imprinting on biological aging pathways is detectable from young ages, suggesting that an effective anti-inflammatory prevention should start from early adolescence and continue among the entire lifespan.

Furthermore, as LTL strongly correlates with markers of chronic but not acute inflammation, LTL can be used as a new valid marker to estimate the long term burden of chronic inflammatory and oxidative stress exposure (75;241;509), information which cannot be obtained by simply measuring circulating levels of CRP,

IL-6 or fibrinogen. This is another important finding of the current thesis, as it provides, for the first time, a potential marker to follow the evolution of chronic inflammation in humans. Indeed, while chronic inflammation is currently considered a major driver of aging and age-related diseases, the lack of a marker which reliably inform on the individuals inflammatory burden, did not allow the correct identification of those individuals at greater risk of unhealthy aging.

Furthermore, the use of LTL as a marker of long term inflammatory burden has another important consequence. Indeed, if long term treatment with anti-inflammatory medications is likely to reduce the risk of unhealthy aging, the effectiveness of these treatment strategies can be monitored by measuring LTL. Similarly to glycated haemoglobin in diabetic patients, the measure of LTL could inform on the ability of anti-inflammatory drugs to reduce the chronic inflammatory burden, marking the inflammatory control over a long period. This characteristic of LTL could be particularly useful in patients with chronic inflammatory diseases. In this case, acute fluctuation in the activity of the underline pathology could result in variable circulating levels of CRP and IL-6 but would hardly impact on the LTL measure.

9 FUTURE WORK

Direct measure of oxidative stress damage to the telomere sequence

Do intra-cellular levels of oxidative stress relate with the amount of oxidative stress damage on the telomere sequence? This may be investigated by combining the use of a new real time PCR technique which allows measuring the amount of oxidative DNA damage in the telomere sequence with flow cytometric techniques which measures the total amount of free radicals produced by a specific cell phenotype. Using the periodontal model, it is possible to explore whether, changes in intracellular oxidative stress concentrations induced by periodontal treatment relates with an acute increase of oxoG on the telomere sequence.

Anti-inflammatory treatments and telomere protection

Although evidence supports the hypothesis that reduction of the baseline inflammatory burden could reduce the risk of age related diseases, it is currently unknown whether these approaches can also reduce the rate of telomere shortening and delay the telomere dysfunction. A long term study, including patient with PD could represent a useful model to explore this hypothesis. Patient could be randomly allocated to intensive and nonintensive treatment. After 1 year, the rate of LTL shortening within the two groups could be measured by FISH. This will allow testing whether the reduction of the inflammatory burden expected in the intensively treated group is associated with a slower rate of telomere attrition, as well as with a lower accumulation of dysfunctional telomeres in different cell subpopulations.

Mitochondrial and cytosolic oxidative stress production: their relationship with telomere oxidative stress damage

Are mitochondrial or cytosolic pathways those which provide the greater contribution to the telomere oxidative stress damage? Several reports suggest mitochondrial generation of oxidative species (which is normally up regulated in the elderly) as the main source of genomic and telomere DNA damage. The availability of flowcytometric probes which selectively measure mitochondrial and cytosolic oxidative stress will allow understanding the relative contribution of these two pathways to the telomere oxidative stress damage. This is remarkable as it has the

potential to identify new possible therapeutic target for a more effective prevention of aging and age-related disease.

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