Telomeres and Coronary Heart Disease

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Scott W Brouilette
Department of Cardiovascular Sciences
University of Leicester

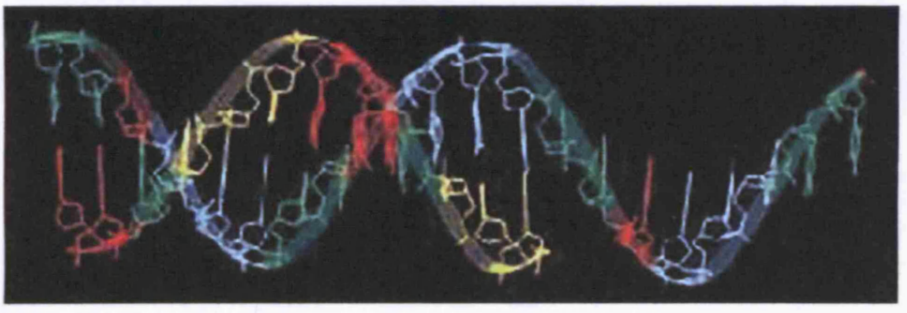
September 2004

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

"Every man desires to live long,

But no man wishes to be old."

Johnathan Swift, author & satirist, 1667-1745.



Acknowledgements

Firstly I would like to thank my supervisor, Professor Nilesh J. Samani, for giving me the opportunity to undertake a Ph.D. in Cardiology. His advice and guidance, particularly when it became apparent that the initial project would have to be stopped, have been greatly appreciated.

Many thanks to Dr. Ravi Singh for his work on the Premature MI and Offspring studies. Thanks to all on the Family Heart Study (FHS), to Dr. Mariuca Vasa-Nicotera and Dr. Massimo Mangino for collaborating on the Telomere-FHS project, to Dr. Richard Cawthon for advice on setting up the PCR-based method of telomere analysis, and to Professor John Thompson for assistance and advice on statistical matters.

I would also like to thank the following people from various locations across Leicester Rachel Boultby, Jenny Clemitson, Sue Adams, Peter Braund, Jasbir Moore and Claire Bodycote.

For providing funding for my research I would like to thank the Medical Research Council, the British Heart Foundation, HeartSearch and the Sir Jules Thorne Charitable Trust.

Thank you to my family for their help (both emotional and financial!) throughout the last few years: Mum, Dad, Gramps, Nan, Cal and Todd. Events have emphasised the importance of family, and shown that sometimes things need to be kept in perspective.

And, finally, to Kellie. Thank you for providing the guiding light that had been missing for so long. I hope you realise just how greatly your love and influence have been felt.

Declaration

I confirm that the work presented in this thesis is my own, except where indicated. In this regard, I would like to specifically acknowledge the following:

The subjects for the premature MI study (Chapter 3) and their clinical characterisation and biochemical measurements were carried out by Dr Ravi Singh, who was investigating the role of platelet reactivity and polymorphisms in risk of MI

The subjects for the Offspring Study (Chapter 4) were recruited by Nashat Qamar and Aqib Bhatti, two intercalated BSc medical students who were studying platelet function in healthy subjects with and without a strong history of premature CHD.

The WOSCOPS Biobank, studied in Chapter 5, was established by the WOSCOPS investigators, and I am particularly grateful to Professor Chris Packard for allowing me access to the samples and related clinical data.

The sib-pairs analysed in Chapter 6 were recruited as part of the BHF Family Heart Study and I am again grateful to the BHF Family Heart Study Investigators for allowing me to access this resource for these studies, and for allowing me access to the microsatellite genotype data they had generated as part of their project.

SW Brouilette
University of Leicester

TELOMERES AND CORONARY HEART DISEASE

Abstract

Coronary heart disease (CHD) is the most common cause of premature morbidity and mortality in the Western world. Although much is known about risk factors that predispose to CHD, several aspects remain unclear. In particular, variation in susceptibility and variation in age of onset. In this thesis I have explored the hypothesis that these aspects are related to inter-individual variation in "biological" ageing. Using mean telomere length as a marker of biological age, I show that:

1. Subjects with premature myocardial infarction (MI) have significantly shorter telomeres than age-sex matched, healthy, controls. The mean telomere length in MI subjects was similar to controls almost 11 years older.
2. Healthy young adult children of families with a strong history of premature MI have shorter telomeres than age matched children of families without such a history.
3. Shorter telomere lengths are associated with increase risk of subsequent CHD events in a prospective study. This analysis was carried out on samples collected in the West of Scotland Coronary Prevention Study (WOSCOPS). This randomised blinded trial was designed to examine the benefits of statin treatment on preventing CHD and showed a 30% reduction of events in those treated with pravastatin. Interestingly, my analysis showed that this benefit of statin is only seen in those subjects at higher risk of CHD based on their telomere length.

As the final part of the thesis I carried out a quantitative linkage trait (QTL) analysis in sib-pairs in an attempt to identify genetic loci regulating telomere length. I report the mapping of a major QTL on chromosome 12 that determines almost 50% of the inter-individual variation in mean telomere length.

These findings support a novel "telomere" hypothesis of CHD. They indicate that telomere biology is intimately linked to the genetic aetiology and pathogenesis of CHD. Specifically, the findings suggest that (i) those individuals born with shorter telomeres may be at increased risk of CHD (ii) rather than individual genes, a more global structural property of the genetic material may explain the familial basis of CHD (iii) variation in telomere length may explain, in part, the variable age of onset of CHD. The findings provide several new avenues for future research.

Publications and presentations from the work presented in this thesis

Publications

Brouilette S, Singh RK, Thompson JR, PhD, Goodall AH, PhD, Samani NJ. White cell telomere length and risk of myocardial infarction. **Arterioscler Thromb Vasc Biol** 2003; 23: 842-846.

Brouilette S*, Vasa-Nicotera M*, Mangino M, Thompson JR, Braund P, Clemitson J-R, Mason A, Bodycote CL, Raleigh SM, Louis E, Samani NJ. Mapping of a major locus determining telomere length in humans. **Am J Hum Genet** 2004 (in press) (*equal contributors)

Presentations

Brouilette S, Singh RK, Thompson JR, PhD, Goodall AH, PhD, Samani NJ. Biological age and risk of premature MI. British Cardiac Society Annual Meeting, Glasgow, 2003. This abstract was short-listed for the Young Investigator's Award and won a runner-up prize.

Brouilette S, Singh RK, Thompson JR, PhD, Goodall AH, PhD, Samani NJ. White cell telomere length and risk of premature MI. European Society of Cardiology Meeting, Vienna, 2003. This abstract was short-listed for the Young Investigator's Award in the Clinical Science section and won a runner-up prize.

CONTENTS

Abbreviations

11BHSD	11b-hydroxysteroid dehydrogenase
ABCT-1	ATP binding cassette transporter-1
ADR	adrenergic receptor
AGE	advance glycation end products
AHA	American Heart Association
ALT	alternative lengthening of telomeres
AME	apparent mineral corticoid excess
ANP	atrial natriuretic peptide
APO	apolipoprotein
ASP	affected sib pair
AT	ataxia telangiectasia
β-gal	β-galactosidase
BHF	British Heart Foundation
BMI	body mass index
BP	blood pressure
CHD	coronary heart disease
cM	centimorgan
CQ	Comparative Quantification
CRP	C-reactive protein
Ct	threshold cycle
CVD	cardiovascular disease
DBP	diastolic blood pressure
DM	diabetes mellitus
DNA	deoxyribonucleic acid
DZ	dizygotic
EDTA	Ethylenediamine tetraacetic acid
EPC	endothelial progenitor cell
EPO	erythropoietin
FA	Fanconi anaemia
FFA	free fatty acid
FH	familial hypercholesterolaemia
FISH	fluorescence in-situ hybridisation
FMD	flow-mediated endothelium dependent dilation
HAEC	human aortic endothelial cell
HD	Huntingdon's disease
HDL	high density lipoprotein
HUVEC	human umbilical vein endothelial cell
IBD	indentical by descent
IBS	indentical by state
ICAM-1	intracellular cell adhesion molecule-1
IDDM	insulin-dependent diabetes mellitus
IL-1	interleukin-1

ILGF	insulin-like growth factor
IMT	intima-media thickness
IVC	inferior vene cava
kb	kilobase
LBW	low birth weight
LDL	low density lipoprotein
LOD	logarithm of the odds
Lp	lipoprotein
LTPA	leisure time physical activity
LVH	left ventricular hypertrophy
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
M-CSP	macrophage colony stimulating factor
MI	myocardial infarction
MLSP	mean lifespan potential
MMP	matrix metalloproteinase
MPV	mean platelet volume
mtDNA	mitochondrial DNA
MWt	molecular weight
MZ	monozygotic
NHEJ	non-homologous end joining
NIDDM	non-insulin-dependent diabetes mellitus
NO	nitric oxide
NOS	nitric oxide synthase
NTC	no-template control
OD	optical density
PAI	plasminogen activator inhibitor
PARP	poly (ADP ribose) polymerase
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PENT	primer-extension-nick-translation
PFGE	pulse field gel electrophoresis
PP	pulse pressure
PWV	pulse wave velocity
QTL	quantitative trait locus
Rn	maximum fluorescence
RNA	ribonucleic acid
ROS	reactive oxygen species
RR	relative risk
RT-PCR	reverse transcription polymerase chain reaction
SA-β-gal	senescence associated β-galactosidase
SBP	systolic blood pressure
SD	standard deviation

SDS	sodium dodecasulphate
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SSC	sodium saturated citrate
STELA	single telomere length analysis
SVC	superior vena cava
TOLA	Telomere oligonucleotide ligation assay
TDR	telomerase-dependent replication
Tdt	terminal deoxynucleotidyl transferase
TERC	telomerase RNA primer
TERT	telomerase reverse transcriptase
TF	tissue factor
TIMP-1	tissue inhibitor of matrix metalloproteinase-1
TNF	tumour necrosis factor
TOLA	telomere oligonucleotide ligation assay
TPE	telomere position effect
TRF	telomere restriction fragment
TRF1	telomere repeat binding factor 1
TRF2	telomere repeat binding factor 2
VCAM-1	vascular cell adhesion molecule-1
VSCM	vascular smooth muscle cell
vLBW	very low birth weight
WBC	white blood cell
WHO	World Health Organisation
WS	Werner's syndrome

INTRODUCTION

1.1 THE HEART & CORONARY CIRCULATION

1.1.1 Cardiac Structure

The heart (Figure 1.1) is the centre of the cardiovascular system. It is situated between the lungs and is a component of the mediastinum, the mass of tissue between the lungs, extending from the sternum to the vertebral column. It is enclosed by the pericardium.

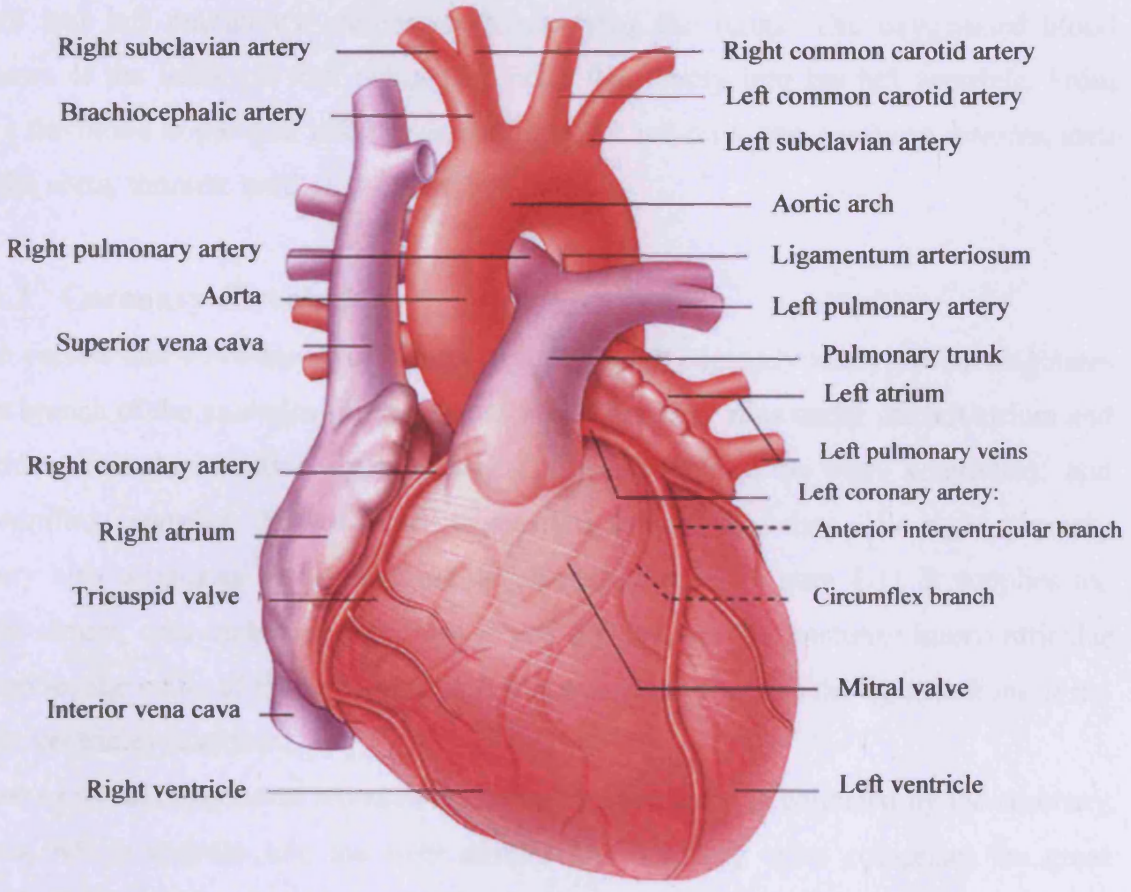


Figure 1.1: Cardiac structure & coronary circulation.
(Image adapted from http://www.tylercvc.com/coronary_circulation.htm)

The wall of the heart is divided into three layers: the epicardium (external layer), the myocardium (middle layer), and the endocardium (inner layer). The myocardium is the cardiac muscle tissue, constitutes the bulk of the heart, and is responsible for the contraction of the heart. The interior of the heart is divided into four chambers, the atria and the ventricles. The atria are separated by the atrial septum, and the ventricles by the

ventricular septum. Externally, the coronary sulcus separates the atria from the ventricles.

The right atrium receives blood from all parts of the body, with the exception of the lungs, and receives blood through three vessels. In general the superior vena cava (SVC) brings blood from parts of the body superior to the heart; the inferior vena cava (IVC) from parts inferior to the heart; and the coronary sinus, which drains blood from most of the vessels supplying the heart. The right atrium then delivers the blood into the right ventricle, which in turn pumps into the pulmonary trunk. This then divides into the right and left pulmonary arteries, both supplying the lungs. The oxygenated blood returns to the heart via four pulmonary veins that empty into the left ventricle. From here the blood is pumped into the ascending aorta and on to the *coronary arteries*, arch of the aorta, thoracic aorta, and abdominal aorta.

1.1.2 Coronary Circulation

The vessels that serve the myocardium include the left coronary artery, which originates as a branch of the ascending aorta (Figure 1.1). The artery runs under the left atrium and divides into the anterior interventricular (supplies blood to both ventricles), and circumflex (supplies the left ventricle and left atrium) branches. The right coronary artery also originates as a branch of the ascending aorta (Figure 1.1). It supplies the right atrium, runs under the right atrium and divides into the posterior interventricular (supplies the walls of the two ventricles) and marginal (supplies the myocardium of the right ventricle) branches.

Most of the deoxygenated blood in the coronary circulation is collected by the coronary sinus, which empties into the right atrium. The coronary sinus comprises the great cardiac vein, which drains the anterior aspect of the heart, and the middle cardiac vein, which drains the posterior aspect of the heart.

1.2 IMPACT OF CARDIOVASCULAR DISEASE (CVD)

Cardiovascular disease (CVD) is the main cause of death in the UK, accounting for over 240,000 deaths a year (Figure 1.2). The main forms of CVD are coronary heart disease (CHD), and stroke. CHD causes over 120,000 deaths a year in the UK: approximately 1 in 4 deaths in men and 1 in 6 deaths in women (British Heart Foundation, 2003). Nearly all deaths from CHD are due to a myocardial infarction (MI).

CHD costs the National Health Service (NHS) approximately £1,750 million a year with hospital care accounting for about 53% and buying and dispensing drugs about 34%. Less than 1% is spent on the primary prevention of CHD. When considered in conjunction with working days lost due to death, illness, and informal care of people with the disease, CHD costs the UK economy approximately £7,055 million a year.

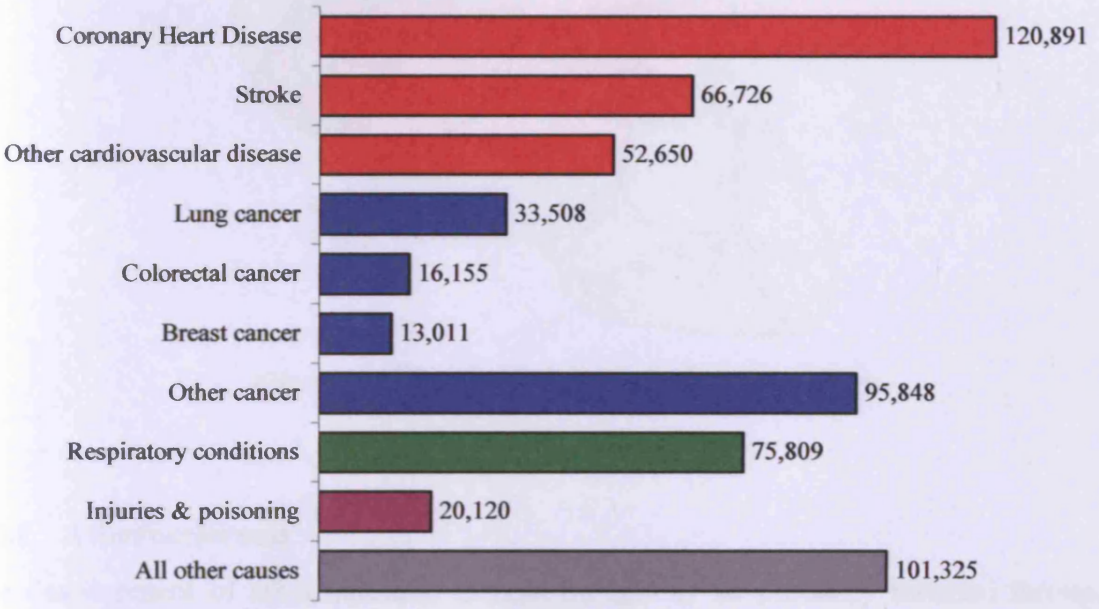


Figure 1.2: **Deaths by cause in the UK, 2001** (British Heart Foundation, 2003). Coronary heart disease, stroke and various other cardiovascular diseases, taken together, accounted for over 240000 deaths in 2003.

1.3 CORONARY HEART DISEASE (CHD)

Coronary heart disease (CHD), also referred to as coronary artery disease (CAD), is a chronic disease in which the coronary arteries become "hardened" and the lumen is narrowed, as shown in Figure 1.3, by a process called atherosclerosis. Atherosclerosis starts at a young age and develops over several decades. For most of the time it remains "silent", but if the luminal narrowing becomes severe it can result in the myocardium not receiving enough oxygen-rich blood, particularly during periods of exertion. This can cause chest pain, pressure and discomfort (angina). Alternatively, if a blood clot

forms abruptly over an area of atherosclerotic tissue, it can occlude the artery, reducing the flow of blood and resulting in necrosis of the muscle (myocardial infarction).

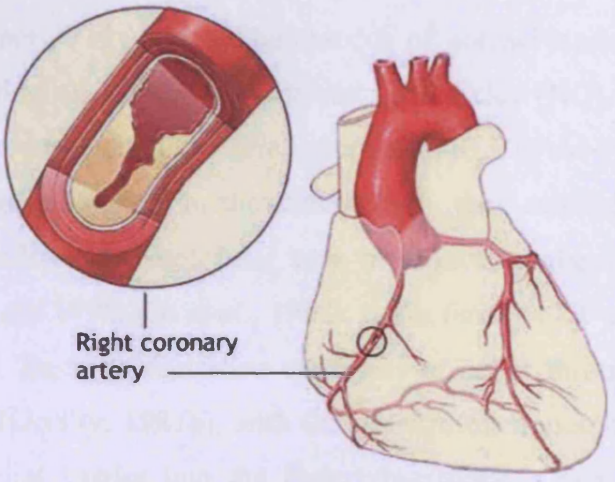


Figure 1.3: Occlusion of the right coronary artery.

1.3.1 Atherosclerosis

The development of atherosclerosis is now thought to be primarily initiated through endothelial damage (reviewed by Ross (Ross, 1999)), caused by the various risk factors for CHD (detailed in Chapter 1.3.2.1). As a result, changes to the endothelium occur that favour atherogenesis.

Enhanced monocyte adherence to the endothelium is an important factor involved in the initiation of atherosclerosis (Huang *et al.*, 1995), as the normal endothelium does NOT generally support binding of white blood cells. Shortly after the initiation of endothelial dysfunction, for example by an atherogenic diet, patches of arterial endothelial cells begin to express adhesion molecules; vascular cell adhesion molecule-1 (VCAM-1) in particular binds the type of lymphocytes found in early human and experimental atheroma, that is monocytes and T-lymphocytes (Libby *et al.*, 2002). This highlights the crucial role that VCAM-1 plays in initiating the development of atherosclerosis, and is supported by the

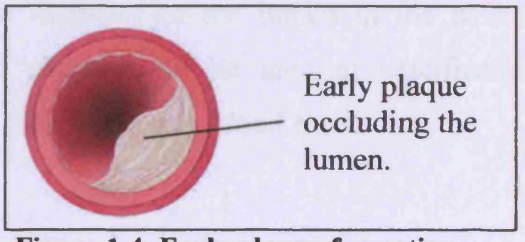


Figure 1.4: Early plaque formation

observation that mice genetically engineered to express defective VCAM-1 show interrupted lesion development (Cybulsky *et al.*, 2001).

Evidence suggests that impairment in the endogenous atheroprotective mechanisms commonly occur at branches in the arterial system where endothelial cells experience disturbed flow (Topper *et al.*, 1996). The absence of normal laminar shear stress may reduce local production of endothelium-derived nitric oxide (NO), a molecule that has anti-inflammatory properties and can limit expression of VCAM-1 (De Caterina *et al.*, 1995). Once lymphocytes adhere to the endothelium, they can then penetrate into the intima, a process mediated by molecules such as monocyte chemoattractant protein-1 (MCP-1) (Boring *et al.*, 1998; Gu *et al.*, 1998), and a family of T-cell chemoattractants (Mach *et al.*, 1999). Intimal penetration continues to occur throughout the course of lesion development (Gerrity, 1981b), with the passage of monocytes and lymphocytes through the endothelial barrier into the underlying tissue. Once into the underlying tissue, monocytes then differentiate into macrophages (Gerrity, 1981a) and ingest oxidised low-density lipoprotein (LDL). Macrophage colony-stimulating factor (M-CSF) augments expression of scavenger receptors for modified lipoprotein particles, resulting in the formation of "foam" cells (Qiao *et al.*, 1997; Smith *et al.*, 1995). The presence of these large, lipid-laden foam cells in intimal lesions is one of the most prominent and consistently found features of atherosclerosis in humans (Geer *et al.*, 1961), and, due to their appearance, these early lesions are typically referred to as "fatty streaks. There is no initial effect on the diameter of the lumen as external remodelling occurs. As the process progresses, an early plaque with a smooth, dome-shaped mass that protrudes into the lumen (Figure 1.4) is formed. From this stage intermediate plaques can form; these plaques then become fibrous, covered by a dense cap of connective tissue with embedded smooth muscle cells, usually overlaying a core of lipid and necrotic tissue (Ross, 1993). Figure 1.5 illustrates the effect of the plaque on the lumen of a coronary artery: compared to the diameter of the lumen in the normal coronary artery (1.5A), the formation of a plaque with an area of calcification effectively halves the diameter of the lumen (1.5B), restricting blood flow.

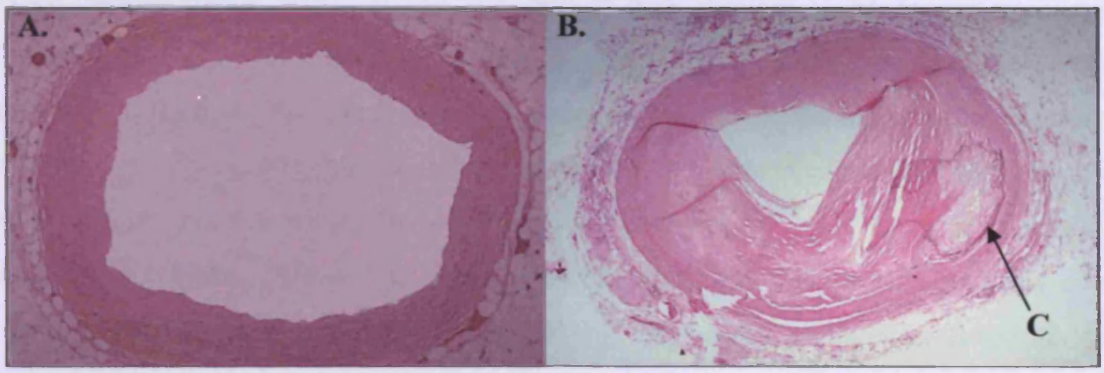


Figure 1.5: Occlusion of a coronary artery. A. Normal coronary artery with no atherosclerosis and an unobstructed lumen (http://medstat.med.utah.edu/WebPath/ATH001.html). **B.** The diameter of the lumen is approximately halved, with an area of calcification (C) visible at the right (http://medstat.med.utah.edu/WebPath/ATHHTML/ATH003.html).

As the inflammatory process continues the activated lymphocytes and arterial cells release fibrogenic mediators, including peptide growth factors, that promote replication of smooth muscle cells (Ross, 1999). At this stage in the development of the atherosclerotic plaque there is an abundance of activated macrophages within the atheroma. These macrophages can produce proteolytic enzymes that are capable of degrading the fibrous cap, leading to an increased susceptibility to rupture. Activated T-lymphocytes release γ-interferon, a factor that also contributes to plaque destabilisation by halting collagen synthesis by smooth muscle cells (Libby *et al.*, 1996; Libby, 2001). In addition to production of proteolytic enzymes, macrophages also produce tissue factor (TF), a pro-coagulant and trigger to thrombosis. Lipid hydroperoxides, lysophospholipids and carbonyl compounds localise in the lipid fraction of the atheroma (Witztum and Berliner, 1998). The apoprotein moieties of the lipoprotein particles can also undergo modification in the artery wall; they become antigenic and are capable of eliciting the response of T-cells (Stemme *et al.*, 1995), a process that activates the antigen-specific adaptive arm of the immune response (Libby *et al.*, 2002).

1.3.1.1 Plaque vulnerability

Traditionally, plaques that appear ulcerative, fissured, and/or thrombotic and that are characterised histologically by a central lipid core, inflammatory infiltrate, and cap thinning have been termed "vulnerable" (Kereiakes, 2003). The vulnerable plaque has been implicated in the development of unstable angina, myocardial infarction, and

sudden cardiac death, although this scenario in now thought to be overly simplistic (Casscells *et al.*, 2003; Maseri and Fuster, 2003). For example, the concept of the vulnerable plaque as the final common pathway by which atherothrombotic events occur fails to explain why plaque inflammation may be present in patients with chronic stable angina and yet absent in some patients who present with an acute coronary syndrome (Kereiakes, 2003). The unpredictability of patient outcomes is probably due, at least in part, to fluctuations in risk factors e.g. day-to-day changes in diet, activity, stress, smoking, infection, hydration and blood pressure (Casscells *et al.*, 2003).

The plaque itself consists of anatomical (central lipid core, thin cap) and functional (intrinsic thrombogenicity, intra-plaque inflammatory infiltrate) components, and is also modified by exogenous factors such as mechanical stress (Yamamoto *et al.*, 2003), infection (Anderson and Muhlestein, 2004), blood viscosity (Sawchuk *et al.*, 1999), and coagulability (Johnstone *et al.*, 1996; Muller and Tofler, 1992). Many plaques progress in an episodic manner due to episodes of thrombosis triggered by rupture, erosion, or occasionally endothelial cell activation or inflammation (Bruschke *et al.*, 1989; Yokoya *et al.*, 1999). Another mechanism of rapid plaque growth is haemorrhage into the plaque, a process central to carotid artery rupture (Burke *et al.*, 1999; Sillesen and Nielsen, 1998). Most plaques that underlie a fatal or nonfatal myocardial infarction are less than 70% stenosed, hence it is vascular biology and not the degree of stenosis that determines plaque stability (Libby and Aikawa, 2003).

1.3.1.2 Plaque rupture

The final common pathway in plaque instability is the predominance of collagen breakdown over collagen synthesis in the fibrous cap. In an unstable plaque, numerous cell types are activated in a process that is very similar to that in other chronic inflammatory conditions, such as rheumatoid arthritis (Pasceri and Yeh, 1999). Smooth muscle cells (SMCs) develop twice the volume of secretory granules as normal quiescent cells (Chen *et al.*, 1997); monocytes become tissue macrophages (Galis *et al.*, 1995), mast cells become positive for tumour necrosis factor -α (TNF-α) (Kaartinen *et al.*, 1996) and T-lymphocytes are also activated (Libby, 1995). The progression of an individual atheroma to stability or instability is determined by a number of cytokines (Barath *et al.*, 1990; Sukhova *et al.*, 1999; van der Wal *et al.*, 1994), and summarised in Table 1.1. For example, TNF-α stimulates production of lipoprotein-trapping

proteoglycans, CSF's cause macrophage replication and γ-interferon suppresses smooth muscle replication (Bobik *et al.*, 1999; Libby, 1995). The activity of metalloproteinases is also important as they digest the collagen present within the fibrous cap, increasing the likelihood of rupture. TNF-α and interleukin-1 (IL-1) upregulate macrophage matrix metalloproteinase (MMP) activity (Kol *et al.*, 1998; Rajavashisth *et al.*, 1999; Saren *et al.*, 1996), and oxidised LDL has been shown to double MMP activity, whereas native LDL has no effect (Bennett *et al.*, 1995; Henderson *et al.*, 1999; Xu *et al.*, 1999). The extracellular protein tenascin-C, which also induces MMP expression and causes SMC apoptosis, is strongly expressed in the unstable plaque, but not in the normal vessel wall (LaFleur *et al.*, 1997; Wallner *et al.*, 1999).

Table 1.1:Summary of The Role of Cytokines

Cytokine	*Main action in unstable plaque*	*Other actions*
TNF-α(Rajavashisth *et al.*, 1999)	Upregulates adhesion molecules	Increases thrombogenicity
Il-1β(Rajavashisth *et al.*, 1999)	Activates endothelial cells	Causes SMC apoptosis
Matrix metalloproteinase (Sukhova *et al.*, 1999)	Digests collagen	Digests elastin
Tenascin(Wallner *et al.*, 1999)	Stimulates MMP expression	Causes SMC apoptosis
Transforming growth factor-β (TGF-β)(Bobik *et al.*, 1999)	Stimulates collagen synthesis	Stimulates lipid-trapping proteoglycans
Tissue factor (Toschi *et al.*, 1997)	Promotes thrombin generation	Promotes MMP expression
Insulin-like growth factor-β (IL-GF-β)(Henderson *et al.*, 1999)	Suppresses collagen expression	Causes SMC apoptosis

1.3.1.3 Thrombus formation

The endothelium usually has anticoagulant properties. Tissue factor (TF) is a potent coagulant, and oxidised LDL (oxLDL) has been shown to induce endothelial cells (Drake *et al.*, 1991; Fei *et al.*, 1993) and monocytes (Brand *et al.*, 1994) to express high levels of TF. Drake *et al.* (Drake *et al.*, 1989) have demonstrated that there is abundant TF in the intima of atherosclerotic lesions. Plasminogen activator inhibitor (PAI) levels are also increased when endothelial cells are exposed to ox-LDL (Latron *et al.*, 1991). Thus, plaque rupture exposes the flowing blood to these elevated levels of TF that result in thrombus formation (Figure 1.6).

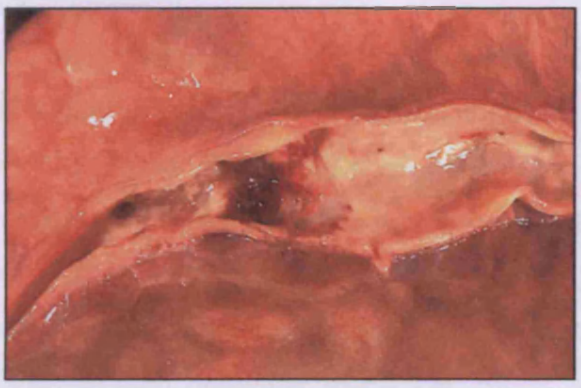


Figure 1.6: An example of plaque haemorrhage. An atherosclerotic plaque displaying haemorrhage, one of the complications of atherosclerosis. Such haemorrhage can acutely narrow the lumen (http://medstat.med.utah.edu/WebPath/ATHHTML/ATH007.html).

Thrombus formation may occur within the intima, resulting in plaque enlargement. At rupture, thrombus does not initially occlude the lumen, but two events may now occur:

1. thrombus totally occludes the functional lumen;
2. thrombus becomes lysed and the fissure reseals.

When rupture followed by resealing occurs, a stable condition is reached, albeit with an increase in the size of the plaque. In unstable angina, plaque fissuring and thrombus formation in the earlier stages are observed. Ultimately, the thrombus may occlude the lumen, resulting in regional infarction.

1.3.2 Aetiology of CHD

The development of CHD is dependent on a number of factors, such as the presence of well-established risk factors (hypertension, smoking, diabetes etc.), the presence of various emerging risk factors (C-reactive protein and homocysteine) and the effect of the genetic background of the individual. In addition, it has also been proposed that nourishment *in utero* can result in growth patterns that predispose to CVD. Thus, the aetiology of CHD represents a complex interaction between various risk factors, overlaid on differing genetic backgrounds.

1.3.2.1 Established risk factors for CHD

Age & gender

Age and sex are two important non-modifiable risk factors for CHD. Over the last 25 years it is clear that the death rates from CHD steadily increase with age, and were higher in men than in women at all age ranges, with rates in both sexes peaking between 65-74 years (British Heart Foundation, 2003).

Serum cholesterol

High serum cholesterol is a major risk factor for development of CHD. The average blood cholesterol level for adult men is about 5.5mmol/l and for adult women about 5.6mmol/l. Mean serum cholesterol increases with age in both sexes, as does the percentage of the population having cholesterol levels above 5.2mmol/l. The percentage above 5.2mmol/l in men peaks at 81.9% between 55-64 years (British Heart Foundation, 2003). In women the percentage (>5.2mmol/l) in any given age range is less than in men, until the 55-64 year block, when the percentage of men begins to decrease, while in women it continues to increase (British Heart Foundation, 2003).

Low density lipoproteins (LDLs) act as the main transport route for cholesterol, serving as a 'donor' to peripheral tissues and the liver (50% of uptake). The concentration of cholesterol carried in LDL (LDL-c, commonly referred to as the "bad" cholesterol) is predominantly dictated by metabolic events in the liver (Dietschy, 1997). LDL's are taken up by both LDL receptor (LDL-r) dependent and independent pathways (Izem *et al.*, 1998). Endocytosis leads to the formation of an endosome with LDL bound to it's receptor inside. Acidification leads to separation with many of the receptors being recycled to the cell surface. Lysosomal enzymes then digest the LDL. Increased free cholesterol concentration due to hydrolysis of LDL-derived cholesterylester has important effects on cholesterol metabolism.

High-density lipoprotein (HDL) is thought to play a role in removing cholesterol from cells and transporting it back to the liver (Dietschy, 1997) and it has been found to facilitate cholesterol excretion by macrophages (Chait *et al.*, 1982; Mahoney *et al.*, 1982). It also provides a degree of antioxidant effect by protecting plasma lipids from peroxidation (Klimov *et al.*, 1993), and high levels of plasma HDL are correlated with reduced atherosclerotic complications (Miller, 1980). The percentage of men having a mean total HDL-cholesterol ***less then*** 1.0 steadily increases with age i.e. as they get older the percentage having less of the "good" cholesterol increases (British Heart Foundation, 2003). In women the levels show no steady trend. Ethnicity also plays a role: the percentage of people from different ethnic groups having HDL-cholesterol levels below 1.0 shows considerable variation; in the general population the value is around 10% but in Indians the value is approximately 28% and in Pakistanis almost 45% (British Heart Foundation, 2003).

Smoking

Smoking increases the risk of developing CHD in men and women (Friedman *et al.*, 1981; Willett *et al.*, 1981). It has been estimated that about 20% of deaths from CHD in men and 17% in women are due to smoking (British Heart Foundation, 2003). Smokers are younger than non-smokers when they suffer their first MI and more of them are males (Landmark, 2001). The increase in risk can be partly explained by the adverse effect of smoking on plasma fibrinogen, platelet function (Erikssen *et al.*, 1977) and lipid profile (Calori *et al.*, 1996; Djousse *et al.*, 2000; Raftopoulos *et al.*, 1999). It may also be accounted for, however, by the acute bradycardia, increase in blood pressure and generalized vasoconstriction accompanying smoking, due to a nicotine-dependent activation of the sympathetic nervous system (reviewed by Omvik (Omvik, 1996) and Benowitz (Benowitz, 1997)). Passive smoking has also been shown to increase the risk of developing acute coronary syndromes (Pitsavos *et al.*, 2002) and CHD (He *et al.*, 1999). Cessation of smoking results in a decrease in risk for CHD and all-cause mortality (Rosenberg *et al.*, 1985; Rosenberg *et al.*, 1990).

Hypertension

Hypertension is the term used to describe a chronic elevation of systemic arterial blood pressure. The American Heart Association (AHA) defines hypertension as arterial blood pressure (BP) higher than 140/90mmHg while the World Health Organisation (WHO) uses 160/95mmHg (Brody *et al.*, 1994), emphasising that a definition in terms of a specific value is largely arbitrary.

Monogenic (Mendelian) forms of hypertension account for less than 1% of cases (Kaplan, 1998), and include Liddle's syndrome (Liddle *et al.*, 1963) and apparent mineralocorticoid excess (AME) (Li *et al.*, 1998; Mune *et al.*, 1995; Wilson *et al.*, 1995). Essential, primary, or idiopathic hypertension is defined as elevated BP in which secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism, or other causes of secondary hypertension or monogenic forms are not present. Essential hypertension accounts for 95% of all cases of hypertension (Kaplan, 1998). Essential hypertension is a heterogeneous disorder, with different patients having different causal factors that lead to high BP.

Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterised by a lack, or relative lack, of insulin, resulting in impaired utilisation of carbohydrates, and altered lipid and protein metabolism. Primary diabetes mellitus is subdivided into insulin-dependent (IDDM, or type I), and non-insulin-dependent (NIDDM) or type II. Diabetic hyperglycaemia leads to oxidative stress in the vessel wall through the formation of advanced glycation end products (AGE) and enhanced binding to the receptors for AGE (Chappey *et al.*, 1997; Schmidt and Stern, 2000). These products can bind specific receptors (RAGE – Receptors for AGE) and augment production of proinflammatory cytokines. The diabetic state also promotes oxidative stress mediated by reactive oxygen species (ROS) and carbonyl groups (Baynes and Thorpe, 1999).

Obesity

CHD is associated with the upper range of body weight, obesity being defined as a relative weight of 140% or greater, or a body mass index (BMI) equal or greater than 30 (National Research Council, 1989). Obesity can contribute to CHD indirectly by affecting insulin resistance (reviewed Bjorntorp (Bjorntorp, 1991)), and the prevalence of diabetes and hypertension is greater among those with excess body weight compared to normal body (Cercato *et al.*, 2004)

BMI is not the only parameter of use when considering obesity; a waist-hip ratio greater than 1.0 in men and 0.8 in women (i.e. denoting an abdominal rather than hip fat pattern) is associated with greater risk of CHD (National Research Council, 1989). Indeed, men in the uppermost quintile of waist-hip ratio are almost three times more likely to develop CHD compared to those in the lowest (Rimm *et al.*, 1995).

Of course, obesity tends to result from both a poor diet and a lack of physical exercise. A meta-analysis carried out in the early 1990's found nearly a two-fold increased risk of death from CHD for individuals with sedentary compared with active occupations (Berlin and Colditz, 1990). A larger study of over 12,000 male subjects focused on leisure time physical activity (LTPA), as assessed by questionnaire. Dividing the amount of LTPA into tertiles, individuals in the lowest tertile had excess mortality rates for all-case mortality (15%), CHD mortality (27%) and cardiovascular mortality (22%), compared to men in the middle tertile (Leon and Connett, 1991).

While physical activity has been shown to reduce the relative risk of both CHD mortality and cardiovascular mortality, the onset of acute myocardial infarction has been associated with heavy physical activity less than one hour prior to the event (Mittleman *et al.*, 1993; Willich *et al.*, 1993). This implies that where there is an underlying problem, attempting excessive physical activity can have severe, or even fatal, consequences. Individuals diagnosed with CHD should embark on a programme of light-to-moderate exercise; brisk walking of more than 1.5 miles per day has been associated with a lower incidence of CHD than those walking less than 0.25 mile per day (2.5% vs 5.1%), and 0.25 - 1.5 miles per day (4.5%) in a cohort of elderly, physically capable men, aged 71-93 years (Hakim *et al.*, 1999). A similar, prospective study of 72,317 nurses found that brisk walking was associated with fewer coronary events (Manson *et al.*, 1999).

1.3.2.2 Other and emerging risk factors

C-reactive protein

Inflammatory components are believed to contribute greatly to instability and rupture of atheromatous plaque leading to athero-thrombotic events (Carr *et al.*, 1997; Pasterkamp *et al.*, 1999; van der Wal *et al.*, 1994).

The C-reactive protein (CRP) pentamer is a marker of the inflammatory process. CRP is present in the vessel wall (Torzewski *et al.*, 2000) and is capable of inducing the expression of the adhesion molecules E-selectin, VCAM-1 and intracellular-cell adhesion molecule (ICAM-1) by endothelial cells (Pasceri *et al.*, 2000). It also acts as a chemoattractant for monocytes (Pasceri *et al.*, 2001), increases monocyte production of TF (Cermak *et al.*, 1993), opsonizes LDL and facilitates entry of native LDL into macrophages via CD32 (Bharadwaj *et al.*, 1999; Zwaka *et al.*, 2001), thus loading macrophages with cholesterol.

CRP has been shown to be higher in CHD cases than controls (Delanghe *et al.*, 2002), and appears to predict poor prognosis in patients with unstable angina (Liuzzo *et al.*, 1994), acute MI (Pietila *et al.*, 1996) or acute stroke (Muir *et al.*, 1999). Increased CRP levels are observed in patients with hypertension (Retterstol *et al.*, 2002), and have been reported in overweight adults (Visser *et al.*, 1999). In a study of 247 patients who had their first MI before the age of 55 (males) or 60 (females), the relative risk (RR) of cardiac death was shown to double with increasing CRP quartiles (Retterstol *et al.*,

2002). It has also been shown that CRP localises in foam cells in atheromatous plaques (Pasceri *et al.*, 2000; Torzewski *et al.*, 2000), and increased CRP levels have been associated with an increase in the *magnitude* of MI (Pietila *et al.*, 1987).

Homocysteine

Homocysteine is an amino acid biosynthesised during metabolism of methionine (an essential amino acid). Circulating levels of homocysteine are normally low due to its rapid metabolism via one of two pathways: 1) a vitamin B_{12} and folate dependent re-methylation pathway that regenerates methionine or, 2) a pyridoxal 5' phosphate (PLP; vitamin B6) dependent trans-sulphuration pathway that converts homocysteine into cysteine.

In 1975 McCully and Wilson (McCully and Wilson, 1975) postulated that moderately elevated plasma total homocysteine (tHcy) concentrations were causally related to the development of CHD. Evidence of a positive association between elevated homocysteine levels and cardiovascular disease has been supported by meta-analysis of retrospective data, which estimated that 10% of all cases of cardiovascular disease could be attributed to elevated concentrations of tHcy (Boushey *et al.*, 1995), and that a rise of 5μmol/l was sufficient to increase cardiovascular risk by 20-30% (Ueland *et al.*, 2000). Furthermore, a recent meta-analysis of prospective studies showed that a 25% lower concentration of tHcy (about 3μmol/L) was associated with an 11% lower risk of ischaemic heart disease and a 19% lower risk of stroke (The Homocysteine Studies Collaboration, 2002).

While the exact mechanism underlying the association between elevated homocysteine and increased risk of CHD has yet to be elucidated, a common C → T transition at nucleotide 677 of the MTHFR gene (an enzyme in homocysteine metabolism) gene has been identified; the TT homozygotes are predisposed to hypercholesterolaemia, particularly in those with relatively low folate intake (Frosst *et al.*, 1995; Klerk *i.*, 2002). However, while some studies have demonstrated an association between the TT genotype and risk of CHD (Klerk *et al.*, 2002; Kluijtmans and Whitehead, 2001; Jee *et al.*, 2000; Morita *et al.*, 1997), there are several others that do not (Brattström *et al.*, 1998; Rotenbacher *et al.*, 2002; Kim *et al.*, 2001).

Left ventricular hypertrophy

Left ventricular hypertrophy (LVH) is an adaptation of the heart to chronic pressure/volume overload; this provides an immediate reduction in wall stress, but in the long term LVH is an important independent risk factor for cardiovascular morbidity and mortality (Kannel, 1983). The hallmark of LVH is hypertrophic growth of the cardiac myocyte as a result of a requirement for increased contractile power. In response to a pressure stimulus there is induction of transcription factor-coding early genes in the left ventricle (Sadoshima *et al.*, 1992), followed by the re-expression of genes that are only otherwise expressed in the left ventricle during foetal development (Brown *et al.*, 1993). An increase in protein content and a reorganisation of contractile elements from a series to a parallel arrangement results in the hypertrophic phenotype.

Alcohol

Evidence suggests that moderate drinkers have lower rates of CHD than abstainers (Manttari *et al.*, 1997), but the effects of moderate alcohol consumption among patients with *established* CHD is unclear. The reduction in risk has also been extended to individuals with type II diabetes in which moderate alcohol consumption is associated with significant risk reduction (Valmadrid *et al.*, 1999) (and reviewed by Tanasescu and Hu (Tanasescu and Hu, 2001)) This may be due to alcohol lowering the production of AGE in diabetics through its metabolite, acetaldehyde (Al-Abed *et al.*, 1999).

Some alcoholic beverages offer greater beneficial effects; red wine polyphenol, for example, has been reported to exert relatively greater benefit through inhibition of platelet aggregation (Wang *et al.*, 2002) (and reviewed by Burns (Burns *et al.*, 2001)). However, heavy alcohol intake, or binge drinking, is associated with increased cardiovascular mortality (Shaper and Wannamethee, 2000), possibly through inhibition of fibrinolysis that persists until the following morning (van de *et al.*, 2001).

Infectious agents

The only intact microbes commonly present in atherosclerotic plaques are herpes simplex virus and *chlamydia pneumoniae* (Leinonen and Saikku, 2002), two agents that are able to initiate and accelerate atherosclerosis in animal models. Lehto *et al* (Lehto *et al.*, 2002) have recently demonstrated an association between chlamydia antibodies with intimal artery calcification, and, after adjustment for other cardiovascular risk factors, a

dose-response relationship between the antibodies and degree of calcification. It has been suggested that *chlamydiae*, when present in the arterial plaque, may release lipopolysaccharide (endotoxic) and heat shock proteins that are capable of stimulating production of proinflammatory mediators by vascular endothelial cells, smooth muscle cells and infiltrating leucocytes (Kol *et al.*, 1999). Any agents causing persistent infection in the vessel wall can directly promote a pro-inflammatory, pro-coagulant and pro-atherogenic environment. However, many studies have failed to show differences in serum antibody levels to any infectious agents (De Backer *et al.*, 2002).

White blood cell count

Higher white blood cell (WBC) counts are a predictor of CHD mortality, independent of the effects of smoking and other traditional CVD risk factors, which indicate a role for inflammation in the pathogenesis of CHD (Hansen *et al.*, 1990).

Mean platelet volume

An increase in mean platelet volume (MPV) is an indicator of larger platelets; larger platelets are metabolically and enzymatically more active than small platelets (Corash *et al.*, 1977). Platelets play a crucial role in thrombus formation, thus alterations to platelet biology may have significant consequences in relation to CHD and subsequent myocardial infarction. For example, platelet aggregation is enhanced in the offspring of young ischaemics (Khalil *et al.*, 1997), and patients with pre-existing coronary artery disease and an increased MPV (> or = 11.6 fl) are at higher risk of MI (Endler *et al.*, 2002).

Increased levels of fibrinogen &, Lp(a)

In the final step of the clotting cascade, thrombin cleaves fibrinogen to produce fibrin. The fibrin monomers spontaneously polymerise to produce the structural backbone of a thrombus. Levels of fibrinogen are elevated in CHD patients (De Backer *et al.*, 2002) and it is now recognised as an independent risk factor for CHD (Heinrich *et al.*, 1994). Lipoprotein (a) (Lp(a)) is also involved in clot formation. Lp(a) concentration has been shown to be higher in patients with CHD compared to controls (Nogues *et al.*, 1992; Schaefer *et al.*, 1994) in both men and women (Vashisht *et al.*, 1992). It is atherogenic

because it can be deposited in the arterial wall and can interfere with fibrinolysis (Angles-Cano *et al.*, 2001) by competing with plasminogen.

1.3.2.3 The genetic basis of CHD

The degree of risk associated with family history has been estimated for various cardiovascular diseases, from a wide variety of studies. One such study of 45,317 male health professionals aged 40-75 years with no diagnosis of CHD with mean follow-up of 1.6 years found that if a parent had an MI before the age of 70, the relative risk (RR) of cardiac death and MI were 2.2 (maternal) and 1.7 (paternal) (Colditz *et al.*, 1991). The incidence of MI was also found to be higher in individuals with a higher incidence of positive family history; among individuals having more than one first-degree relative with a history of MI the incidence of MI was 31%, compared to 15% for controls (Ciruzzi *et al.*, 1997). Perhaps the most compelling evidence has come from twin studies. For example, a study of 21,000 Swedish twins found that among men, the RR (of death secondary to CHD) if the twin died of CHD before the age of 50 were 8.1 (monozygous) and 3.8 (dizygous) (Marenberg *et al.*, 1994a). Among women, RRs were 15.0 and 2.6 respectively.

While a shared environment may explain part of the tendency for CVD to occur in families, much of the work over recent years has focussed on identifying candidate genes involved in various processes that may contribute to the overall risk profile. Thus, polymorphisms in a vast number of genes have been studied, and their association with various CV diseases determined.

Lipid metabolism

Familial hypercholesterolaemia (FH) is a common autosomal codominant hereditary disease affecting approximately 1:500 in the Caucasian population (Goldstein *et al.*, 2001). The condition is caused by defects in the LDL-r gene, and one of the most common characteristics of affected subjects is premature CHD. However, the age-of-onset of CHD as a result of FH has been shown to depend on specific alleles of the ATP binding cassette transporter 1 (ABCA1) gene; the R219K allele appears to delay the onset of CHD in patients with FH (Cenarro *et al.*, 2003). Decreased plasma LDL particle size has also been associated with premature CAD (Austin *et al.*, 2000; Campos *et al.*, 1992; Koba *et al.*, 2000), and it has been suggested that the Trp64Arg variant in

the β3-adrenergic receptor gene may be associated with a reduction in LDL particle size (Okumura *et al.*, 2003).

Apolipoprotein E (apo E) contributes to the reverse transport of cholesterol to the liver. There are three common variants of the aopE gene: E3, E2 and E4. The E4 allele is associated with increased, and the E2 allele with decreased, plasma apolipoprotein B-containing proteins such as LDL (Davignon *et al.*, 1998). Consequently, carriers of the E4 allele tend to have higher risk, and carriers of the E2 allele have lower risk, of CHD (Wilson *et a*l., 1996).

Haematological & inflammatory factors

As the degree of thrombus formation following plaque rupture will determine the severity of the clinical manifestation, polymorphisms that affect the clotting cascade may exert considerable influence. The Pl(A2) polymorphism of the glycoprotein IIIa subunit of the fibrinogen receptor (GPIIb-IIIa) is associated with an increased risk of CHD (Burr *et al.*, 2003), and the TT genotype of the Factor XII (FXII) 46C>T polymorphism is associated with a high risk of CHD in men with high cholesterol (Zito *et al.*, 2002). In addition, the thrombospondin-2 polymorphism (T>G substitution in 3'-untranslated region), is associated with a reduced risk of premature MI (Boekholdt *et al.*, 2002).

As we have already seen, inflammation is a key process in atherosclerosis. TNF-α is a key inflammatory cytokine, and the presence of a single nucleotide polymorphism (SNP) at the promoter of TNF-α (-308), in patients type II diabetes mellitus, is associated with an odds ratio for CHD of 2.86 (Vendrell *et al.*, 2003).

Other polymorphisms

As discussed in Chapter 1.3.1.2, plaque rupture can result from degradation of the fibrous cap by MMPs, so it is unsurprising that a polymorphism affecting the expression of these proteinases has been investigated. For example, a common functional 5A/6A polymorphism in the promoter of the stromelysin-1 (MMP3) gene has been identified. Compared to the 5A/5A genotype, the relative risks (of fatal. and non-fatal MI and sudden coronary death) for the 5A/6A and 6A/6A genotypes have been shown to be 1.37 and 3.02 respectively (Humphries *et al.*, 2002). A further study has indicated that individuals carrying the 6A/6A genotype may be predisposed to developing

atherosclerotic plaques with significant stenosis, whereas those carrying the 5A allele may be predisposed to developing unstable plaques (Beyzade *et al.*, 2003).

A polymorphism in the human β1-adrenergic receptor (ADRB1) is associated with acute MI (Iwai *et al.*, 2003), another in the ScaI atrial natriuretic peptide (ANP) may be associated with nonfatal MI and the extent of CAD (Gruchala *et al.*, 2003), and the T(-786)-->C mutation in the endothelial nitric oxide synthase (eNOS) gene decreases insulin sensitivity (Yoshimura *et al.*, 2003).

Considering just the few examples here we get an indication of the way in which polymorphisms in seemingly unrelated biological systems can interact to alter the risk of development and progression of CHD.

1.3.2.4 The "Barker" Hypothesis

In the late 1980's a totally different hypothesis was put forward to try and explain the risk of developing CHD. David Barker and colleagues suggested that an individual's nourishment *in utero* and in infancy results in patterns of foetal and infant growth that somehow "programmes" the development of various risk factors that are key determinants of CHD (Barker, 1989). The association between low birth weight and CHD has since been demonstrated in various studies across the world (Barker, 1989; Eriksson, 2001; Frankel, 2003; Leon, 1998; Rich-Edwards, 1997; Stein, 1996), and it appears that the mechanisms responsible result from low birth weight combined with a period of increased ("catch-up") growth during early childhood (Barker *et al.*, 2002; Eriksson *et al.*, 2003a; Eriksson *et al.*, 2003b). This most likely results in alterations to organs such as the pancreas (Phipps *et al.*, 1993) and liver (Barker *et al.*, 1992; Barker *et al.*, 1993), that are involved in processes such as glucose metabolism, cholesterol metabolism and haemostasis. Consequently, a number of alterations in certain processes may contribute, perhaps exerting a synergistic effect, to increase the risk of CVD from a very early age. Barker proposes that CHD develops through a series of interactions: the effects of genes are conditioned by foetal growth (Eriksson, 2003), effects of small body size at birth are conditioned by growth during childhood (Eriksson, 2001), and by living conditions in childhood (Barker, 2002) and adult life (Barker, 2001).

While this hypothesis provides a novel explanation for both the development and progression of CHD, it is not without its critics: an editorial in the British Medical Journal (Paneth and Susser, 1995) pointed out that in the work published by Barker *et*

al., no actual measure of nutritional intake in mothers or babies was ever made; nutrition was inferred indirectly from foetal and infant growth. Also, twins have greatly restricted growth in the third trimester and, according to the developmental hypothesis, should experience increased risk of CVD and all-cause mortality. However, it has been shown that mortality among surviving twins differs little from that among the general population (Christensen *et al.*, 1995).

1.3.3 Summary

While we now know a great deal about the complex aetiology of CHD, including an increased understanding of the growing number of recognised risk factors and markers, and the important contribution of the genetic background, there are still many aspects of the disease that remain unexplained. Novel hypotheses, such as the Barker Hypothesis, may provide new insights into the relationship between genetic background and environmental effects; however, fundamental questions regarding the aetiology remain.

There are many cases when individuals may demonstrate very similar profiles in terms of risk factors for CHD, but some go on to develop the disease, while others remain disease-free for many years. It is also apparent that even when such individuals do indeed have identical risk profiles, and subsequently develop CHD, the precise age-of onset can vary considerably. This leads us to question the underlying mechanism that may explain 1) why some individuals seem to exhibit a degree of protection against a set of risk factors that result in the development of the disease in others, and 2) why the age-of-onset is so variable in the presence of similar risk factors. It is possible that the two phenomena described are unrelated, and are the result of differing mechanisms and pathways. Alternatively, they could represent differing manifestations of the same underlying problem.

The incidence of cardiovascular disease is known to increase with advancing age, for a number of reasons. These include increases in blood pressure, reduced distensibility of major vessels, and alterations to the mechanical properties of the vascular endothelium. Given that age-related changes to various biological systems can contribute to the pathogenesis of CHD, one possibility is that differences in the rates of biological ageing in these systems between individuals may explain the observations outlined above. In the presence of identical risk factors, the individual experiencing accelerated biological ageing would be more likely to develop CHD. Similarly, an individual with accelerated

biological ageing may also experience cardiovascular problems at a much younger age than someone ageing at a "normal" rate, but who lives to an old age. In both cases the cardiovascular system is likely to be in the same, dysfunctional, state when the disease is manifest, but has simply occurred earlier in the first person.

In order to more fully understand the potential implications of premature biological ageing, it is necessary to first introduce a number of key theories of biological ageing and to explain how biological ageing differs from chronological ageing.

1.4 THE BIOLOGY OF AGEING

1.4.1 Chronological & Biological Ageing

Chronological ageing is an extrinsic variable that proceeds at the same rate in all individuals of every species; the units of measurement range from days to years to decades. In contrast, biological ageing is an intrinsic variable and, unlike chronological age, does *not* proceed at the same rate in all individuals within a species. Figure 1.7 illustrates the point: some individuals may age at a slower biological than chronological rate (green line), while others may age at a faster biological than chronological rate (red line), resulting in "premature" ageing. The process of ageing *per se* is not a disease. Hayflick (Hayflick, 2000) has observed that disease processes can be distinguished from age-related changes on the basis of at least four criteria. Unlike any disease, age-related changes:

- occur in every animal that reaches a fixed size in adulthood;
- take place in virtually all species;
- occur in all members of a species only after the age of reproductive success;
- occur in animals removed from the wild and protected by humans.

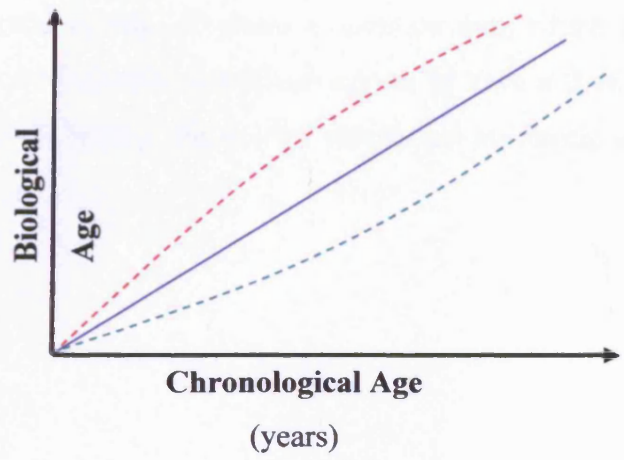


Figure 1.7: Chronological versus Biological age. The relationship may be linear (blue line), however, biological ageing can proceed at either a slower rate (green line), or at a faster rate (red line) than chronological ageing.

Theories of Biological Ageing

Ageing is a process that occurs after reproductive maturation and results from the diminishing energy available to maintain molecular fidelity (Hayflick, 2000). Antagonistic pleiotropy, the hypothesis originally proposed by GC Williams (Williams, 1957), was formulated to explain, in genetic terms, the extension of principles of natural selection, under which reproductive pressure is the defining force, to the post-reproductive period. This hypothesis translates into a "trade-off" between early and late-life fitness, a concept that has been extended in Kirkwood's "disposable soma" theory (Kirkwood, 1977). This theory proposes that ageing is largely the outcome of investment in reproduction, rather than maintenance and repair of the soma. In other words, the balance between metabolic energy needed to maintain and repair the soma and the energy devoted to reproduction accounts for differences in lifespan among species, and, perhaps, variations within members of the same species. This appears to be confirmed in studies that demonstrate that women with fewer children, and women who bear children later on in life exhibit greater longevity (Perls *et al.*, 1997; Westendorp and Kirkwood, 1998), a phenomenon that may be due to the effects of oestrogen. Oestrogen exerts a vasoactive effect, and oestrogen replacement therapy may actually lower blood pressure in post-menopausal women (Akkad *et al.*, 1997; Beljic *et al.*, 1998; Manhem *et al.*, 1998) through modification of homeostatic processes including the renin-angiotensin system (Brosnihan *et al.*, 1997; Gallagher *et al.*, 1999). Aviv (Aviv, 2001) has proposed a more satisfactory explanation for the effect of oestrogen on blood pressure: the presence of oestrogen in the ***pre***-menopausal period retards biological ageing, while its absence in the ***post***-menopausal period accelerates biological ageing. But what is the mechanism(s) responsible for biological ageing?

While a number of different hypotheses have been put forward in an attempt to explain biological ageing, all share a common aim, which is to try and explain why, in the absence of disease, organisms appear to have a finite life span. In general, theories of biological ageing fall in two categories: stochastic and developmental-genetic (Table 1.2).

Table 1.2: Theories of Biological Ageing

Stochastic	*Developmental-genetic*
Somatic Mutation and DNA Repair	Longevity Genes
Error-Catastrophe	Accelerated Ageing Syndromes
Protein Modification	Neuroendocrine
Free Radical (Oxidative Stress) /	Immunologic
Mitochondrial DNA	Cellular Senescence
	Cell Death

Adapted from Troen (Troen, 2003).

Stochastic theories

Stochastic theories propose that ageing is caused by random damage to essential molecules, eventually resulting in sufficient damage to cause physiological decline. The major example is the somatic mutation theory, which proposes that genetic damage from background radiation causes mutations that impair function (Henshaw *et al.*, 1947), ultimately resulting in death (Failla, 1958; Szilard, 1959). The DNA repair theory is a more specific example, based on the observation that the ability to repair this radiation-induced DNA damage in cultured cell lines derived from species with a variety of different life-spans correlates directly with maximum lifespan potential (MLSP) (Hart and Setlow, 1974).

There is a constant turnover of protein within the human body, such that errors occurring in these proteins are simply replaced. The error-catastrophe theory proposes that random errors in synthesis may occur in the very proteins that synthesize DNA (Orgel, 1963) resulting in errors in the molecules they produce. This would lead to amplification in the number of error-containing molecules. In addition, the accumulation of post-translationally modified proteins may impair cellular and, eventually, organ function(s). An example of this, specific to cardiovascular disease, is the reaction of carbohydrates with amino groups of proteins (glycation) giving rise to advanced glycation end-products (AGEs). These AGEs increase with age and AGE cross-links contribute to arterial stiffening in humans (Airaksinen *et al.*, 1993).

It has been proposed that oxidative stress causes most of the age-related changes seen due to molecular damage caused by free radicals as production of free radicals in the heart, kidney and liver has been found to be inversely proportional to the maximum lifespan (Harman, 1956; Harman, 1981). Reactive oxygen species (ROS) may also contribute to somatic accumulation of mitochondrial DNA (mtDNA) mutations,

resulting in a positive feedback loop: mtDNA damage leads to defective mitochondrial respiration, which enhances free radical formation, leading to additional mtDNA damage. Mitochondrial DNA is maternally transmitted, thus, if the oxidative stress/mitochondrial DNA theory of ageing were correct, longevity would be maternally determined. Korpelainen *et al.* (Korpelainen, 1999) have indeed found that heritability estimates for life-span based on mother-offspring regressions are higher than estimates based on offspring-father regressions, thus lending support to the hypothesis.

Development-genetic theories

Developmental-genetic theories propose that ageing is simply part of the genetic program of development and maturation. While there is much evidence in many species that MSLP is under genetic control, the degree of heritability is probably less than 35% (reviewed by Finch and Tanzi, 1997 (Finch and Tanzi, 1997)). There are relatively few genes implicated in longevity in humans; however, the epsilon 4 allele of (ApoE), which is associated with increased CHD, is inversely correlated with longevity (Schachter *et al.*, 1994), and a linkage analysis study has implicated the presence of a gene(s) on chromosome 4 that are associated with exceptional longevity (Perls *et al.*, 2002). Interestingly, the authors accept that such genes may affect susceptibility to disease, rather than altering intrinsic ageing *per se*.

Accelerated ageing syndromes may help to elucidate the genetics basis of the ageing process. The genetic diseases Hutchinson-Guilford syndrome, Werner's syndrome (WS) and Down's Syndrome all display features of accelerated ageing. While a gene showing significant similarity to a DNA helicase has been implicated in WS (Yu *et al.*, 1996), the precise molecular basis for the accelerated ageing remains unclear. Of the animal models that exhibit many of the ageing phenotypes seen in humans, perhaps the most interesting is the *klotho* mouse. This model suffers from a defect in the *klotho* gene, resulting in a syndrome that resembles human ageing, including a short lifespan, infertility, arteriosclerosis, skin atrophy and osteoporosis (Kuro-o *et al.*, 1997). Interestingly, a recent study has demonstrated that the level of serum klotho in a population aged 0-91 years declined in an age-dependent manner (Xiao *et al.*, 2004).

The neuroendocrine theory of ageing proposes that reduced neuronal function, and the consequential effect on their associated hormones, is central to the ageing process (Dilman, 1981; Dilman *et al.*, 1986). Similarly, the immunological theory is based on

the observations that the functional capacity of the immune system decreases with age (reviewed by Pawelec *et al* (Pawelec *et al.*, 1999)).

Cellular senescence is also regarded as a developmental-genetic theory of ageing. Replicative senescence is defined as a state of irreversible growth arrest. In contrast, quiescence is a reversible growth state that can be induced in dividing young cells by serum starvation or contact inhibition. Only a few markers can distinguish between the two states; immunohistochemically detectable β-galactosidase (β-gal) is the most commonly used senescent cell-specific marker (Dimri *et al.*, 1995). Senescent cells appear to accumulate with age in human tissues; β-gal has been shown to be expressed by several human cell types upon senescence in culture (Dimri *et al.*, 1995) and this activity has been termed senescence-associated β-gal (SA-β-gal) and is expressed by senescent cells, but not quiescent, terminally differentiated or immortal cells. Senescent cells are viable and metabolically active; they are still able to respond to environmental signals (Chang and Chen, 1988; Hornsby *et al.*, 1986; Rittling *et al.*, 1986), although they fail to proliferate in response to physiological mitogens. The senescent cell stably, and essentially irreversibly, arrests growth with a G1 DNA content (Campis and Dimri GP, 1996). Failure to proliferate is not due to breakdown in growth factor signal transduction, rather a subset of mitogen-inducible genes are repressed in senescent cells. These include genes that encode many of the enzymes needed for DNA replication, which are normally induced just before the start of S phase. Several phenotypic changes occur during the senescence of almost all cells, including cell enlargement, increased lysosome biogenesis, and decreased rates of protein synthesis and degradation (Stanulis-Praeger, 1987). Additional, cell-type specific changes in gene expression are also observed, for example senescent human fibroblasts and endothelial cells over-express interleukin-1α (IL-1α) and senescent endothelial cells overexpress the adhesion molecule I-CAM (Maier *et al.*, 1990; Maier *et al.*, 1993). These changes function as markers that allow the occurrence of senescence to be observed. The senescence hypothesis of ageing is supported by studies that have demonstrated limited *in vitro* lifespan in glial cells (Ponten *et al.*, 1983), keratinocytes(Rheinwald and Green, 1975), vascular SMCs (Bierman, 1978), lens cells (Tassin *et al.*, 1979), endothelial cells (Mueller *et al.*, 1980) and lymphocytes (Tice *et al.*, 1979). Essentially, the cells undergo a limited number of population doublings before entering senescence.

It is unlikely that ageing is the result of just one of the mechanisms outlined above, rather each contributes to a varying degree, resulting in the geno- and phenotypic changes that are characteristic of the ageing process. However, cellular senescence is perhaps the most widely studied mechanism, providing a link between limited replicative capacity and biological ageing.

Limited Replicative Capacity

During the 1920's Alexis Carrel, a French Nobel-prize-winning surgeon, suggested that explanted cells are immortal and are only unable to replicate continuously because researchers were unsure how to best cultivate the cells. This was based on work carried out on chicken heart fibroblasts in which the cells apparently grew continuously for 34 years (Carrel and Ebeling, 1921). Hayflick has since speculated that the observations may have been the result of an experimental error; he then went on to demonstrate the ***finite*** replicative capacity of normal human fibroblasts and linked this to cellular ageing (Hayflick, 1965; Hayflick and Moorhead, 1961). The initial experiment carried out by Hayflick and his colleague, PS Moorhead, was very simple: equal numbers of normal human male fibroblasts that had divided 40 times were mixed with female cells that had divided only 10 times (unmixed populations served as controls). When the male control population stopped dividing the mixed cells were examined and only female cells found. Hayflick and Moorhead interpreted these observations as the older male cells *remembering* that they are older, even in the presence of younger cells (Hayflick and Moorhead, 1961). They were also satisfied the observations were not due to culture artefacts. Despite some initial difficulties in getting their work accepted for publication the concept of the Hayflick limit (i.e. the number of divisions a given cell type is capable of) is now generally accepted (Shay and Wright, 2000). But this does little to explain what controls the number of divisions a particular cell type is capable of, or how the 'older' cells in the Hayflick and Moorhead experiment *knew* that they were older.

Eukaryotic Cell Replication

An overview of the cell cycle is shown in Figure 1.8. The cycle consists of essentially two stages; the 'M phase' and interphase. During the M phase mitosis and cytokinesis divides the nucleus and cytoplasm. This is the shortest part of the cycle. Interphase, which accounts for approximately 90% of the cycle, consists of three periods of growth:

G_1, S and G_2. Chromosomes are duplicated only during the S (DNA synthesis) phase; growth occurs during G_1 and G_2.

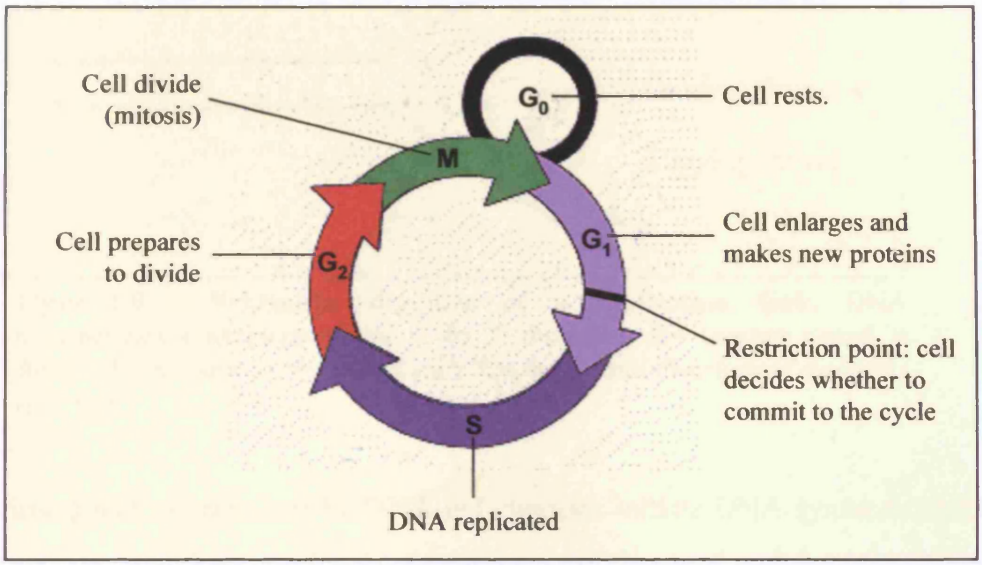


Figure 1.8: Stages of the cell cycle.

Mitosis occurs in 5 stages: prophase, prometaphase, metaphase, anaphase and telophase. The G_1 phase of the cycle is critical as a checkpoint occurs late in the stage, just prior to S-phase. This is the point at which the decision to replicate the cell is made. Assuming all the conditions are favourable the cell divides; otherwise it enters a non-dividing state called the G_0 phase. The onset of the S phase commits the cell to continue through the G_2 and M phases and divide.

A DNA polymerase catalyses the synthesis of DNA; the replication is mediated by the intricate and coordinated interplay of a variety of proteins. The polymerase enzyme adds deoxyribonucleotides to the 3'hydroxyl terminus of a pre-existing DNA chain. The synthesis of new DNA is closely coupled to the unwinding of parental DNA, occurring simultaneously at the 'replication fork' (Figure 1.9). At the fork both strands of parental DNA serve as templates. The overall direction of synthesis is 5'→3' for one strand and 3'→5' for the other. As DNA polymerases only synthesize in the 5'→3' direction there is a 'leading' and a 'lagging' strand. The discontinuous assembly of the lagging strand enables 5'→3' polymerisaztion at the nucleotide level to give overall growth in the 3'→5' direction.

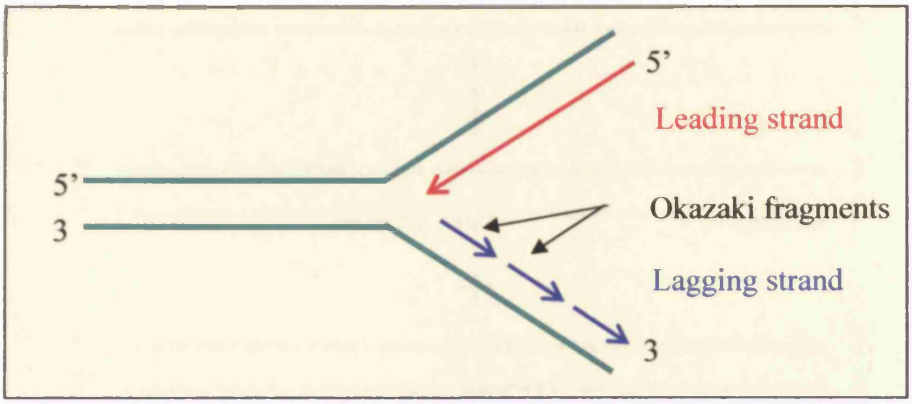


Figure 1.9: Schematic diagram of a replication fork. DNA polymerases synthesize in the 5' to 3' direction; the lagging strand is formed from Okazaki fragments such that the overall direction of synthesis is 3' to 5'.

Short primers that are required by DNA polymerases initiate DNA synthesis. After the polymerase has completed synthesis, the primers are removed and the gaps 'filled' by DNA ligase. Olovnikov was one of the first researchers to notice a problem with this mechanism, and in 1973 he proposed a theory of 'marginotomy' - the shortening of a DNA replica with respect to the template (Olovnikov, 1973). DNA ligase can only *join* two strands together; at the very end of a linear molecule of DNA there will be only one strand, thus the final DNA replica will be shorter then the template by the length of the primer (Figure 1.10).

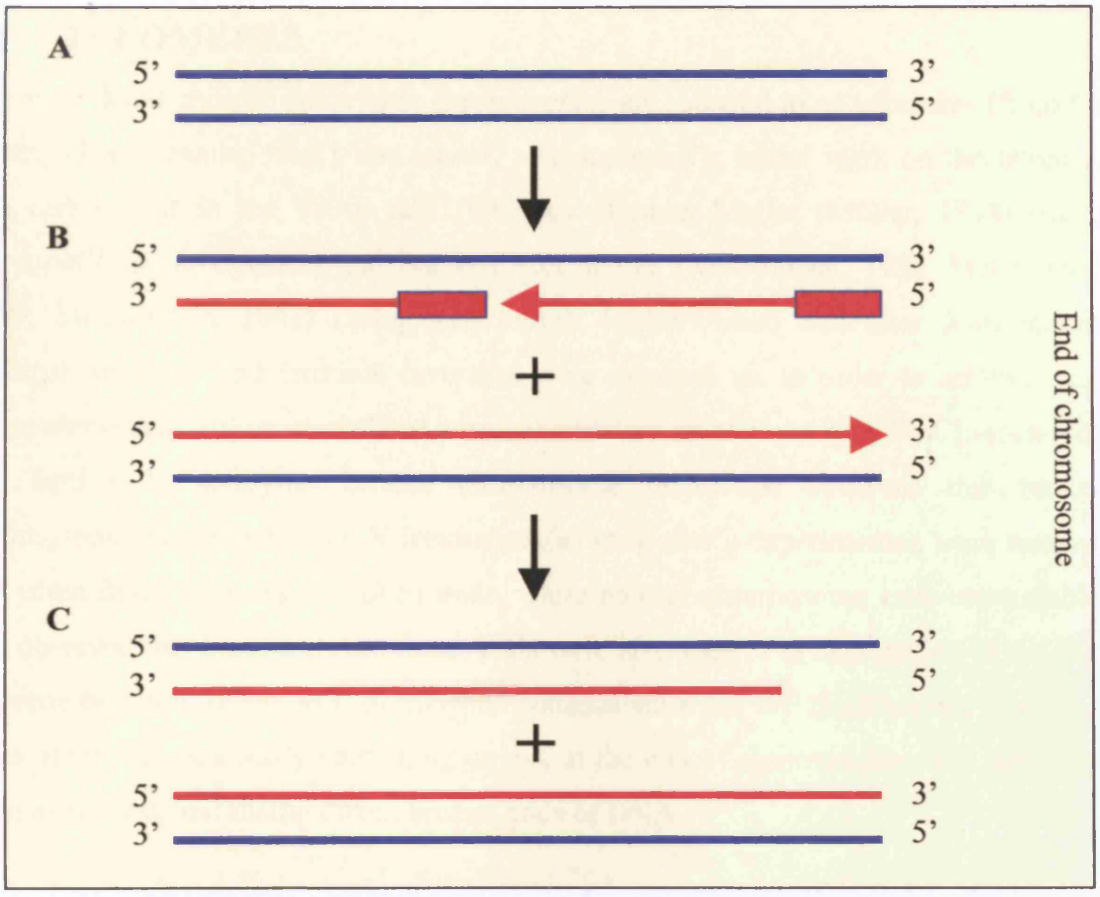


Figure 1.10: The End-Replication Problem. DNA replication by a conventional polymerase towards the end of the chromosome. **A.** Parental DNA. **B.** Bottom strand synthesized by leading strand synthesis (5'→3') to the last nucleotide. Top strand synthesized by discontinuous lagging strand synthesis, primed by RNA primers (red boxes). **C.** RNA primers removed and filled in. A 5' gap is left, as there is now no primer to be filled in.

This is known as The End Replication Problem, and forms the basis of the theory of the **mitotic clock**. A further understanding of the mitotic clock and how it may relate to the concept of biological ageing requires an understanding of the structure of the distal ends of chromosomes, referred to as **telomeres**.

1.5 TELOMERES

The very distal ends of eukaryotic chromosomes are referred to as telomeres (from the Greek *telos*, meaning 'end', and *meros*, 'a component'). Initial work on the telomere was carried out in the 1930s and 1940s by Herman Muller (Muller, 1938) (using *Drosophilla melanogaster*) and Barbara McClintock (McClintock, 1938; McClintock, 1939; McClintock, 1941) (using *Zea mays*). Muller found that after X-irradiation terminal deletions and terminal inversion were rare and so, in order to achieve such chromosome stability, a specialised terminal structure must be present. McClintock took this further by studying broken chromosome ends; she observed that broken chromosome ends induced by X-irradiation (as in Muller's experiments), were reactive and often fused with other broken ends, while natural chromosome ends were stable. She observed that broken chromosome ends were also subject to degradation in the cell, or recombination, often with deleterious consequences for the chromosome involved. Thus, there was obviously something unique at the end of chromosomes that identified them as natural, and distinct from broken ends of DNA.

1.5.1 Basic Structure

Repeat arrays

Each end of a chromosome consists of a block of simple telomeric sequences (Table 1.3), which are tandemly repeated all the way to the end of the chromosome. Telomeric DNA has a strand composition asymmetry resulting in a G-rich and a C-rich strand. The G-rich strand is always orientated 5' to 3' towards the end of the chromosome. The composition of the region is highly conserved, which suggests that both arrangement and composition are critical for telomere function.

Table 1.3: Species-specific telomeric sequence

Organism	*Sequence*
Homo sapiens	AGGGTT
Arabidopis	AGG/AGTTT
S. cerevisae	$G_{1\text{-}3}T$
Plasmodium	AGGGTT(T/C)
Trypanosoma	AGGGTT

1.5.2 Human Telomeres

The number of terminal repeats at a telomere is variable. In man the length of the repeat is shorter in somatic tissues (approx 10kb) than in sperm (approx 15kb); initial mammalian telomere length has been estimated to be in the region of 17kb (De Bono, 1998). Not all of the telomere is double-stranded; the very distal end comprises a single-stranded G-rich telomeric overhang, which in some human cells has been shown to be 150-200bp (Makarov *et al.*, 1997; McElligott and Wellinger, 1997). In human chromosomes, telomeres are adjoined centromerically by a sub-telomeric region consisting of degenerated telomeric DNA sequences and unique repeats (Brown *et al.*, 1990), as shown in Figure 1.11.

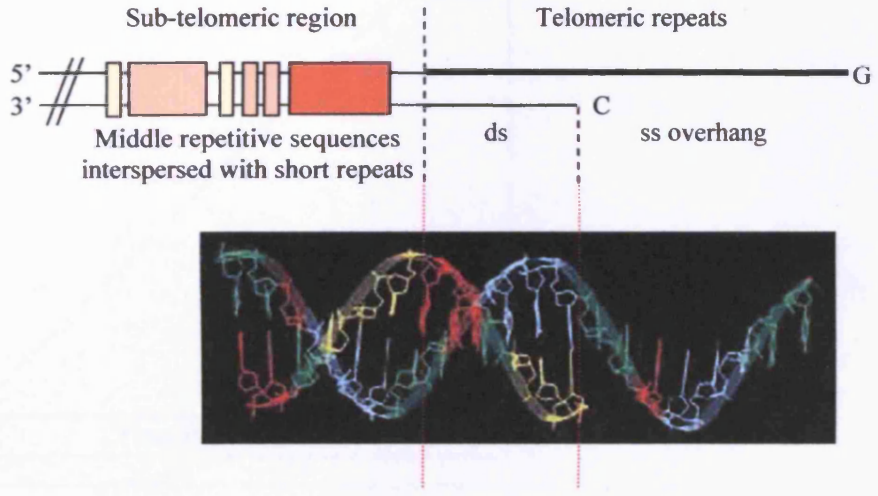


Figure 1.11: Telomeric structure. Schematic representation of a human telomere (upper) and the simplified 3D structure (lower).

1.5.2.1 Telomere length and replicative senescence

As illustrated in the previous section, the direct result of the end-replication problem is that the distal ends (the telomeres) of eukaryotic chromosomes progressively shorten with each round of cell division, with important implications for cellular senescence. A number of cell cycle checkpoints exist, including the mortality phase I checkpoint (M1), at which normal human somatic cells enter replicative senescence and stop dividing. Interestingly, cells transfected with short G-rich single-stranded DNA oligonucleotides designed to mimic exposed telomere ends undergo a p53-dependent cell-cycle arrest and display other M1-like phenotypes (Saretzki *et al.*, 1999). These findings suggest

that M1 is induced when the telomeric structure is compromised, exposing the telomere to DNA damage signalling pathways (de Lange, 2001). Thus, it would appear that when the telomere shortens to a critical length the structure is indeed compromised, inducing senescence, a concept that will be discussed in detail in subsequent sections.

Figure 1.12 shows how, in normal somatic cells, the M1 checkpoint defines the Hayflick Limit i.e. the point at which cells are no longer able to divide. Occasionally mutations and transformation events allow cells to escape M1 and acquire an extended life-span. Cells eventually undergo crisis at mortality phase 2 (M2) and rare mutations can allow just a few clones to escape M2 and become immortal.

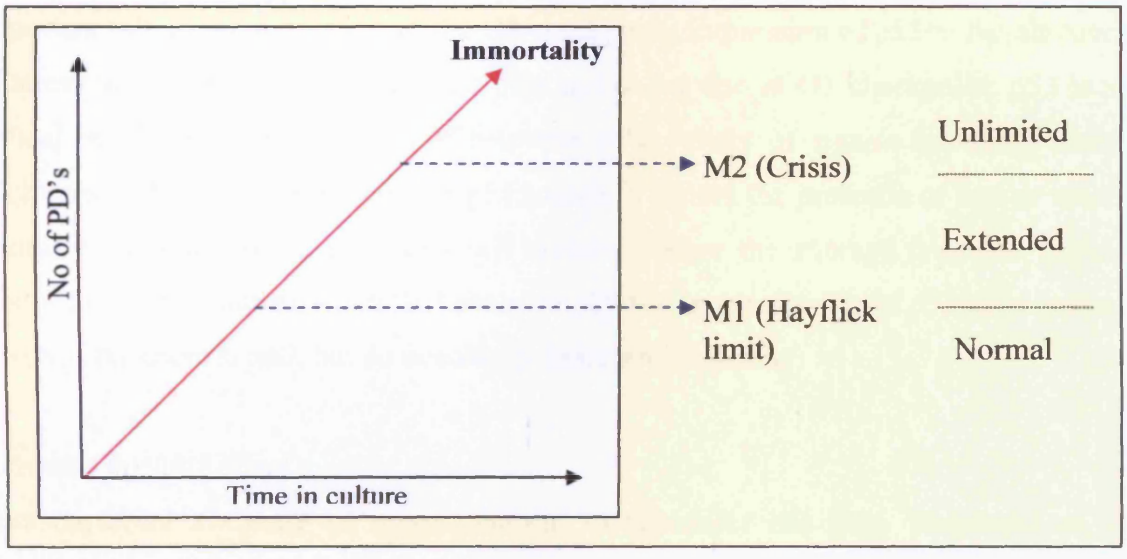


Figure 1.12: Extended lifespan. Cells normally reach M1 (Hayflick limit) and stop dividing. Mutations may allow the cell to escape M1, but they eventually undergo crisis at M2. Very rare mutations may allow a cell to escape M2 and become immortal.

Importantly, there are additional checkpoints that also appear to be influenced by telomere length (Table 1.4).

Table 1.4: Cell cycle checkpoints

Checkpoint	*Stage of cycle*	*Cycle arrested if:*
G1/S	Entry into cell cycle	Cell growth or environmental conditions are inappropriate for division.
G2/M	Entry into mitosis	DNA replication is incomplete or if the DNA is damaged.
M/G1	Metaphase-to-anaphase transition.	Chromosomes are not attached correctly to the mitotic spindle.

The G2 checkpoint is regulated by the activation of multiple pathways that act in concert to inhibit the activity of the cyclin B1/cdc kinase complex, primarily through phosphorylation of cdc2 (Jin *et al.*, 1996; Rhind *et al.*, 1997). p53 also plays an important role in the regulation of the G2 checkpoint. Expression of p53 in the absence of stress induces cell-cycle arrest, not only at G2 but also at G1 checkpoint. p53 is a critical regulator of the senescence response to a variety of signals including short telomeres: it has been proposed that p53 somehow senses the presence of one or more critically short telomeres and halts cell division before the average telomere length erodes to much less than 4-7kb (Itahana *et al.*, 2001). In the absence of p53 cells ignore the short telomere signal, but do eventually cease proliferation.

Telomere position effect

Transcriptional silencing of genes adjacent to telomeres has been postulated as a potential mechanism of telomere-mediated senescence (Wright and Shay, 1992). This phenomenon is referred to as telomere position effect (TPE), and is best characterised in the budding yeast *Saccharomyces cerevisiae*, where reversible silencing of a gene near a telomere appears to be dependent on both telomere length and distance from the telomere (Tham and Zakian, 2002), and marker genes inserted less than 4kb from telomeric repeats were frequently repressed and replicated late in S phase (Dubrana *et al.*, 2001). It is therefore plausible that telomere shortening in humans could progressively affect the expression of numerous genes. Until recently there has been little evidence for TPE in humans, however Baur *et al.* (Baur *et al.*, 2001) have now demonstrated transcriptional silencing near human telomeres. Luciferase reporters were placed adjacent to telomeric repeats and were found to be expressed on average tenfold lower than luciferase reporters at non-telomeric sites; expression was restored by trichostatin A, a histone deacetylase inhibitor (histone deacetylation plays an important

role in modifying chromatin structure and regulating gene expression). To rule out the possibility that the effect was the result of a mixed population of Hela clones or a mutation in the reporter gene, further experiments were also carried out. Over-expression of hTERT in the *telomeric* clones resulted in telomere extension and a concomitant 2-10-fold decrease in luciferase compared with control clones. This observation highlights the "silencing" effect of the longer telomere, with strength of silencing correlated with telomere length. However it has been pointed out (Ning *et al.*, 2003) that these experiments involve genes that are artificially positioned upstream of a telomere and no intact natural sub-telomeric region is present. It remains to be seen how TPE operates on natural telomeric genes that may be hundreds of kilobases from the telomere.

1.5.2.2 Telomere maintenance by telomerase

Mechanisms do exist that enable telomeres to be extended, thus delaying the onset of senescence. Telomerase activity was first identified in *Tetrahymena* (Greider and Blackburn, 1985). It is a ribonucleoprotein enzyme containing both a highly conserved reverse transcriptase (TERT) (Lingner *et al.*, 1997; Nakamura *et al.*, 1997) and an RNA primer (TERC) (Greider and Blackburn, 1989; Singer and Gottschling, 1994) that provides the template for the telomeric repeats that are synthesized at the ends of the chromosomes. Telomerase preferentially binds to and elongates telomeric sequence primers over non-telomeric sequences. In telomerase-dependent replication (TDR) there is telomerase-mediated G-strand elongation with the RNA moiety of the enzyme acting as the template, and the 3' end of the chromosome acting as the primer. However, the complimentary C-rich strand is generated by lagging-strand synthesis, and so a single-stranded overhang will still be created (Figure 1.13).

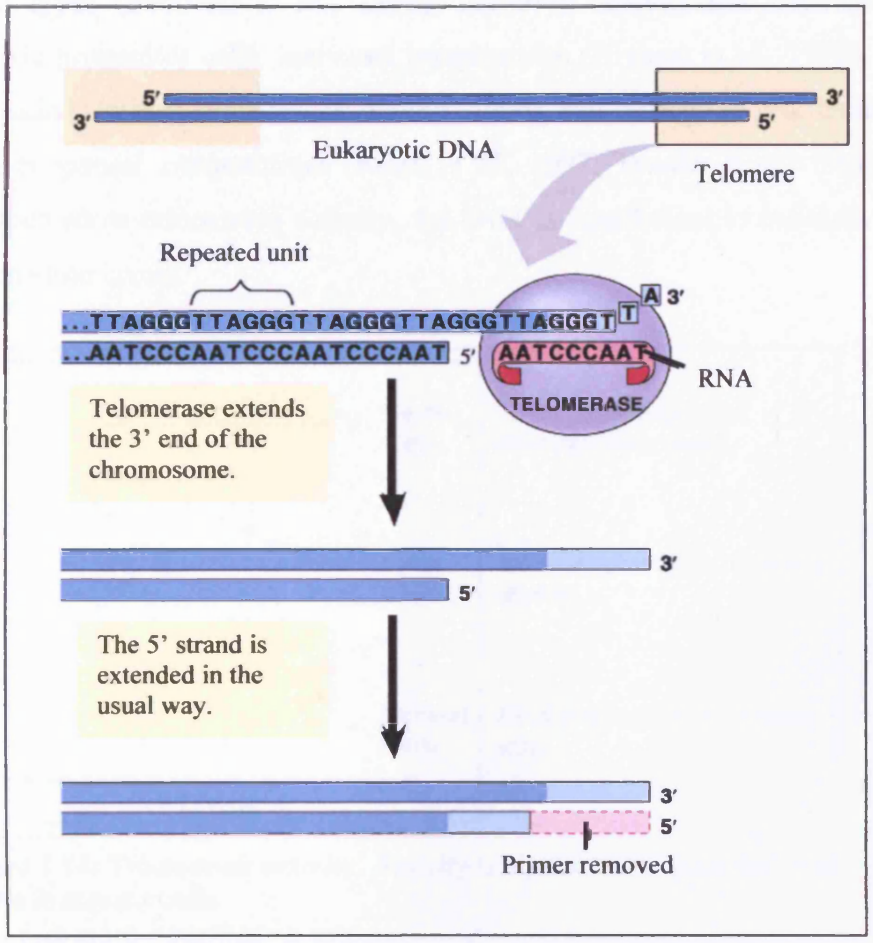


Figure 1.13: Telomerase-dependent replication. Adapted from www.oxy.edu/.../Bio130S_2002/Images/Ch16/fig16_19b.JPG

Telomerase activity

The level of telomerase expression differs markedly between different cell types. Ulaner and Giudice (Ulaner and Giudice, 1997) examined human foetal tissues of 8-21 weeks gestational age for telomerase activity. All tissues expressed telomerase at the earliest ages examined; lung, liver, spleen, and testis maintained telomerase activity through to 21 weeks. Brain and kidney telomerase activity was present up to the 16th week and was undetectable thereafter. Heart tissue did not display activity beyond the 12th week. Lysates of heart, brain, and kidney without telomerase activity failed to inhibit the activity of known telomerase-positive cells, suggesting that suppression of telomerase activity during gestational development is due to a lack of active telomerase rather than to the presence of an inhibitor.

Unlike germ cells and early embryonic cells, *most* somatic cells switch off the activity of telomerase after birth (Figure 1.14). However, activity has been reported in some

somatic cell types (Broccoli *et al.*, 1995): low-level activity has been detected in haematopoietic progenitor cells, activated lymphocytes (Hiyama *et al.*, 1995), the basal layer of the skin (Harle-Bachor and Boukamp, 1996), and during the proliferative phase in the pre-menopausal endometrium (Brien *et al.*, 1997; Tanaka *et al.*, 1998). While these cell types show telomerase activity, the level is insufficient to maintain telomere length at germ-line levels.

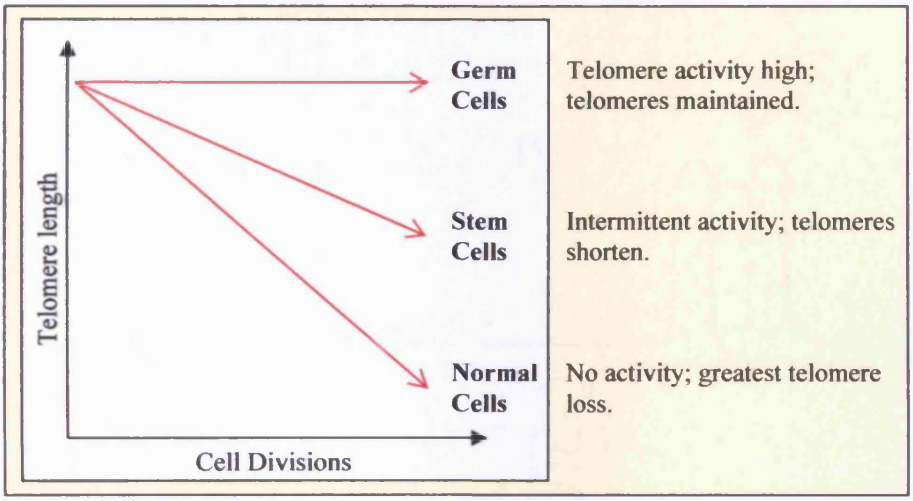


Figure 1.14: Telomerase activity. Activity is highest in the germ line, and lowest in somatic cells.

1.5.2.3 The telomeric complex

A functional telomere is built up through the recruitment, by the underlying DNA, of telomere-specific proteins, including tankyrase, TRF (TTAGGG repeat factor) 1 and TRF 2. It is also becoming clear that the precise conformation of the telomere is as important as its absolute length. Both the G-rich and the C-rich telomeric strands are able to form a number of structures *in vitro* (Figure 1.15). The G-rich strand can adopt a 4-stranded G-quadruplex structure involving planar G-quartets (Sen and Gilbert, 1988; Sundquist and Klug, 1989), and the C-rich strand can form a structure referred to as an i-motiff (Gehring *et al.*, 1993). Although the presence of G-quadruplexes has been demonstrated in the macronuclei of ciliates (Schaffitzel *et al.*, 2001), it is believed that telomeric repeats predominantly form a Watson-Crick double helix under physiological conditions, and that the G-quadruplex and I-motiff may be formed under different conditions, such as high temperature or low pH (Phan and Mergny, 2002).

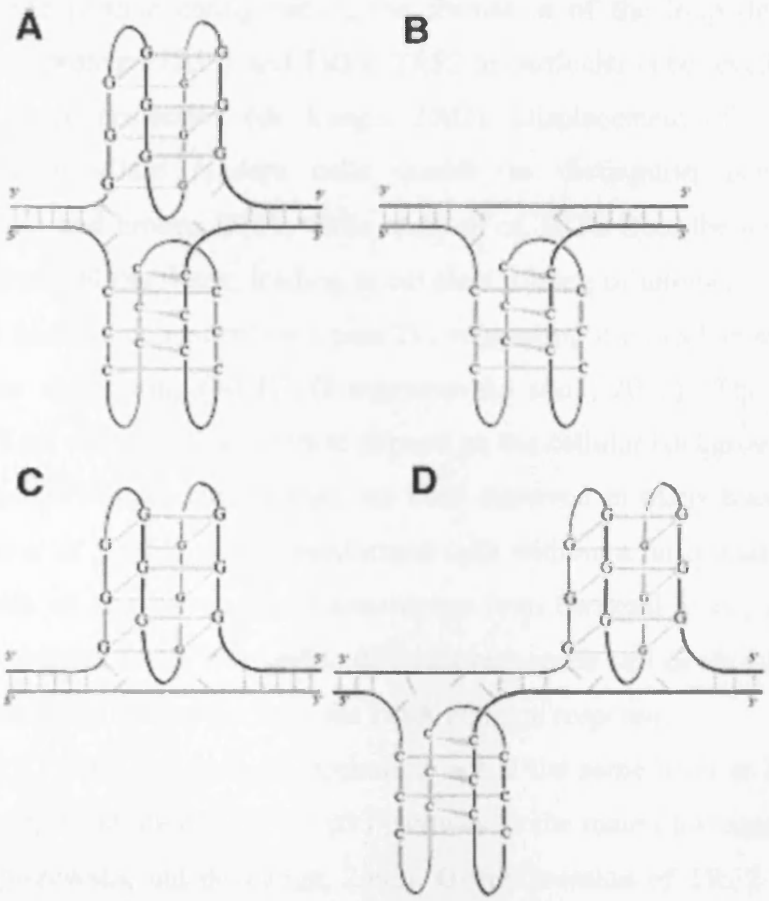


Figure 1.15: Possible configurations of G-quadruplexes and i-motiffs. A) G-quadruplex and an i-motiff at the same location. **B)** An i-motiff. **C)** G-quadruplex. **D)** i-motiff and G-quadruplex at different locations. (Taken from Phan & Mergny, 2002).

While the G-quadruplex and i-motiff configurations are unlikely under normal physiological conditions, an alternative configuration involving both the double-stranded and single-stranded regions of the telomere has been demonstrated *in vivo* (Griffith *et al.*, 1999). It is believed that the so-called ***t-loop*** is formed by the G-rich single strand invading the preceding double-stranded region. Invasion would generate a D-loop consisting of three strands: two G-rich, and one C-rich strand. It has also been reported that a section of the terminal duplex may also invade (Stansel *et al.*, 2001), resulting in a D-loop consisting of four strands: two G-rich and two C-rich. In this case there are a number of possible configurations, including double and triple helices, G-quadruplexes and i-motiffs.

Regardless of the precise configuration, the formation of the loop depends on the telomere-binding proteins TRF 1 and TRF2. TRF2 in particular is believed to be the key protein in telomere protection (de Lange, 2002). Displacement of TRF2 using a dominant negative allele renders cells unable to distinguish between natural chromosome ends and broken DNA, while removal of TRF2 from the telomere results in loss of the G-strand overhang, leading to covalent joining of telomeres (van Steensel *et al.*, 1998), a process dependent on ligase IV, suggesting the mechanism involved is non-homologous end joining (NHEJ) (Smogorzewska *et al.*, 2002). The overall effect of loss of TRF2 on cell growth appears to depend on the cellular background. ***Apoptosis*** in an ATM- and p53-dependent manner has been observed in many transformed cell-lines (Karlseder *et al.*, 1999), while transformed cells without a functional p53 response enter arrest, with all the hallmarks of ***senescence*** (van Steensel *et al.*, 1998). Thus, removal of a protective factor can lead to different outcomes: cell death via apoptosis or senescence, both seeming to stem from the DNA damage response.

Removal of TRF2 from the telomere appears to act at the same level as DNA damage caused by ionising irradiation, with the p53 pathway as the main downstream signalling cascade (Smogorzewska and de Lange, 2002). Overexpression of TRF2 decreases the length at which cells stop dividing from 7kb to 4kb, suggesting that TRF2 is able to 'reset' the senescence point, defined as the telomere length that causes a cell to initiate a senescence pathway (Karlseder *et al.*, 2002). It has also been observed that cells overexpressing TRF2 accumulate fewer chromosomal aberrations than expected based on their shorter telomeres i.e. excess TRF2 is able to protect short telomeres (Karlseder, 2003). The mechanism by which increased numbers of TRF2 molecules protect telomeres could be due to more efficient formation and/or increased stability of the t-loop.

G-overhangs are required for t-loop formation (Griffith *et al.*, 1999; Stansel *et al.*, 2001) (Figure 1.16) and are most likely created by nuclease attack of the 5' strand, recessing it to leave the G-rich 3' strand exposed. It has been proposed that the enzymatic processes involved are tethered to the telomere by TRF2, and that increased amounts of TRF2, therefore, lead to more efficient chromosomal end-processing and overhang formation, which in turn is required for t-loop formation (Karlseder, 2003). This hypothesis could explain how over-expression of TRF2 is able to stabilise shorter telomeres, that would

otherwise induce senescence (Karlseder *et al.*, 2002), and suggests that the length of the overhang may be as important as the length of the entire telomere.

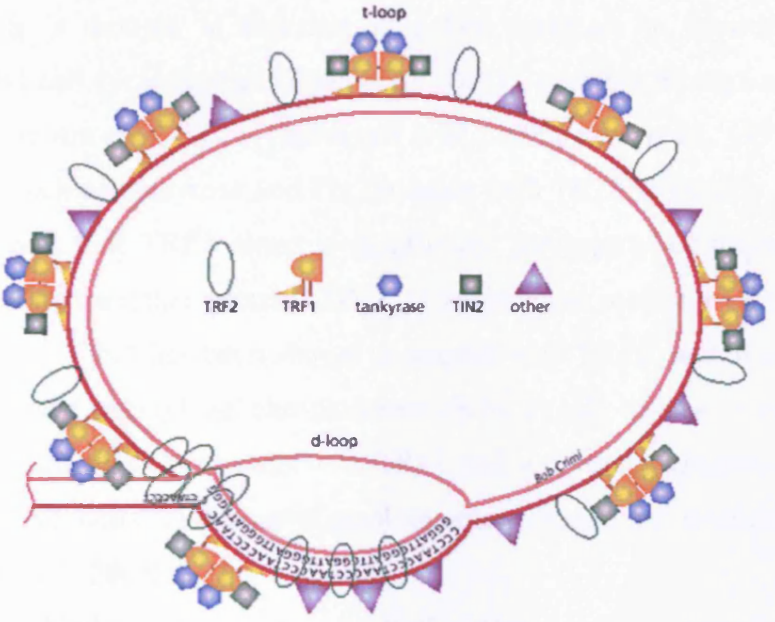


Figure 1.16: Telomere-binding proteins and the t-loop
(biology.yonsei.ac.kr/ PMP/tbp.htm)

Tankyrase is a telomeric poly(ADP-ribose) polymerase (PARP) that binds TRF1. Both tankyrase and TRF1 function as acceptors for adenosine diphosphate (ADP)-ribosylation; ADP-ribosylation of TRF1 diminishes its ability to bind telomeric DNA *in vitro* (Smith *et al.*, 1998). It has also been proposed that tankyrase-mediated ADP-ribosylation of TRF1 opens the telomeric complex, allowing access to the enzyme telomerase (Smith and de Lange, 2000), and that TRF1, when bound to duplex telomeric DNA, blocks extension by DNA polymerases (Smucker and Turchi, 2001). These observations suggest that poly (ADP-ribosyl)ation may regulate telomere function in human cells.

Tankyrase localises with TRF1 at the ends of human chromosomes in metaphase and interphase, and localises to additional sub-cellular sites in a cell cycle dependent manner (Smith and de Lange, 1999). It has also been shown to act as a novel signalling target of mitogen-activated protein kinase (MAPK) (Chi and Lodish, 2000), as well as having a role in cytoplasmic signal transduction pathways (Lyons *et al.*, 2001). Ku70, Ku86, and DNA-Pkcs, components of the DNA-dependent protein kinase (DNA-PK) complex,

have also been shown to interact with telomeric DNA (Bianchi and de Lange, 1999; Hsu *et al.*, 1999), and are proposed to interact with both TRF1 and TRF2 (Bailey *et al.*, 1999; Samper *et al.*, 2000). Pin2 also localises with TRF1 to form the Pin2/TRF1 complex, which is thought to function as a key molecule in connecting telomere maintenance and cell cycle control (Zhou *et al.*, 2003), possibly through mitotic spindle regulation (Nakamura *et al.*, 2001; Nakamura *et al.*, 2002; Shen *et al.*, 1997).

While proteins such as tankyrase and Pin2 interact with TRF1 to modify its function, it has been proposed that TRF1 alone is insufficient for control of telomere length in human cells and that another protein, TIN2, is an essential mediator of TRF1 function (Kim *et al.*, 1999). TIN2 has been shown to interact with TRF1, and to colocalise with TRF1 in nuclei and metaphase chromosomes (Kim *et al.*, 1999). It has since been suggested that human TIN2 interacts with TRF1 and suppresses telomere elongation in telomerase-positive cells, and may control telomere length by modulating telomere structure (Kim *et al.*, 2003).

A new telomere-binding protein, human Pot1 (hPot1), has recently been identified based on homology to the ciliate single-stranded end binding proteins (Baumann and Cech, 2001), and the homologous human protein has also been identified. These Pot1 (protection of telomeres) proteins each bind the G-rich strand of their own telomeric repeat sequence, consistent with a direct role in protecting chromosome ends. A recent study has suggested that hPot1 may bind the 3' end of human telomeres and stabilise them whilst in the 'open' configuration, during various stages of the cell cycle (Colgin *et al.*, 2003). Over-expression of hPot1 could then compete for the 3' overhang, disrupting the t-loop and leaving more telomeres in the 'open' state.

In addition to extending telomeres, it is now believed that telomerase can also protect telomeres by helping to functionally cap chromosome ends; the presence of functional telomerase allows cells to remain competent for proliferation, even with short telomeres (and with no net elongation of short telomeres observed) (Chan and Blackburn, 2002). Thus, active telomerase allows telomeres to remain functional even at lengths that, in the absence of telomerase, would have caused cells to stop dividing or led to telomere-telomere fusions. Recently two general models attempting to explain this action of telomerase have been considered. Chan and Blackburn (Chan and Blackburn, 2002) reviewed evidence suggesting that telomerase may physically interact with the telomere at a critical period in the cell cycle, beyond the time just required for polymerisation,

and protect the exposed single-stranded terminus. However, the authors appear to favour an alternative model in which telomerase may have a proliferation-promoting effect on cells, possibly through some form of signalling to cell-cycle machinery. They suggest that telomerase 'reassures' cells with short telomeres that the enzyme is present, and so telomere integrity will be maintained.

Thus, it is clear that the levels of expression of these proteins are important for the correct function of telomeres. For example, over-expression of TRF1 affects mitotic progression, inducing apoptosis in cells containing short telomeres, but not in cells with long telomeres (Kishi *et al.*, 2001). Dysfunction of TRF1 expression has also been implicated in a subset of acute leukaemia (Ohyashiki *et al.*, 2001). Another study has recently shown that 1) TRF1 and TRF2 mRNA's were greater in normal cells than in human malignant hematopoietic cell lines or in patients with acute leukaemia, 2) human TERT mRNA expression showed changes paralleling telomerase activity, and 3) initially low expression of TRF1 and TRF2 mRNA increased during differentiation (Watanabe, 2001). These studies serve to highlight the importance of the expression levels of the binding proteins, and the impact they have on maintenance of telomeric structure.

1.5.2.4 Factors influencing telomere length

Telomere length is an important factor in maintaining a structure capable of protecting the distal ends of the chromosomes. In the absence of telomerase activity the telomeres shorten as a function of cellular turnover, resulting in replicative senescence. Thus, the length of the telomere is a significant factor in determining the replicative potential of a cell, but what factors influence telomere length?

Telomere length synchrony

Youngren and colleagues (Youngren *et al.*, 1998) have shown that there is remarkable synchrony in telomere length among organs of the human foetus during the gestational period (15-19 weeks), but significant variations in length between foetuses. The synchrony between organs most likely reflects the activity of telomerase exhibited by human foetal tissues (Wright *et al.*, 1996; Youngren *et al.*, 1998), while the difference observed between foetuses may point to differing levels of telomerase expression in

different individuals in the germ-line, in addition to differences in inherited telomere length.

Inheritance of telomere length

Twin studies have demonstrated that telomere length is a heritable trait (Graakjaer *et al.*, 2004; Jeanclos *et al.*, 2000; Slagboom *et al.*, 1994). The results of these studies (examining 115, 49 and 7 twin pairs respectively) have led to an estimate for heritability of approximately 78%. It has also been shown that individuals possess a chromosome-specific pattern of telomere lengths that is similar in homologues from the same donor (Graakjaer *et al.*, 2003; Martens *et al.*, 1998)(Graakjaer *et al.*, 2004), and it has been suggested that this may have been defined in the zygote (Graakjaer *et al.*, 2004)

Telomere attrition

Telomeres in somatic cells are shorter than telomeres in the germ-line, consistent with the end-replication problem and limited replicative capacity. The measured length will be a product of the initial length (at conception), and the replicative stress placed upon that cell during life. The replicative stress results in different rates of attrition in different cells types, leading to a divergence in telomere lengths between tissues that turnover at a higher rate, and those that turnover at a lower rate.

The rate of telomere attrition can also be influenced by other factors. Oxidative damage is repaired less well in telomeric DNA than elsewhere in the chromosome, and oxidative stress accelerates telomere loss, whereas antioxidants decelerate loss (von Zglinicki, 2002). Homocysteine has been shown to increase the amount of telomere length lost per population doubling (Xu *et al.*, 2000).

Sex-related differences in telomere length

Telomere length is generally longer in women than in men (Benetos *et al.*, 2001; Cawthon *et al.*, 2003; Jeanclos *et al.*, 2000). Given that, *in vivo*, some telomerase activity is present in subsets of proliferative somatic cells, and the enzyme is stimulated by oestrogen (Gao *et al.*, 2003; Williams *et al.*, 2001), the longer telomeres observed in women may reflect a slower rate of age-dependent telomere attrition in pre-menopausal women than in men. An oestrogen response element exists on the catalytic sub-unit of the enzyme (Kyo *et al.*, 1999), and there are oestrogen receptors present in vascular

cells (Mendelsohn and Karas, 1999). It is possible that oestrogen-mediated increases in telomerase activity during the menstrual cycle may attenuate telomere attrition in various tissues, including the vasculature. The gender difference observed in humans is mirrored in rodents: a recent study showed that male rats had shorter telomeres than females in all organs examined except the brain, where the lengths were similar (Cherif *et al.*, 2003). These findings indicate that telomeres in rat kidney, liver, pancreas and lung shorten in an age-dependent, gender-dependent, and tissue-specific manner.

1.5.3 The Telomere Hypothesis of Cellular Ageing

Given that, in the absence of telomerase, telomeres shorten with each round of celleular division until they reach a critical point at which they are no longer able to replicate, it is reasonable to postulate that it is the length of the telomere that determines the life-span of the cell. Indeed, the telomere hypothesis of cellular ageing was first proposed by Harley in 1992 (Harley *et al.*, 1992). The theory proposed that telomere length is the **mitotic clock** that regulates cellular life span. Support for the telomere hypothesis comes from studies that have examined telomere length as a function of age: two of the most widely studied cell types, with regard to telomere biology, are fibroblasts and lymphocytes. The mean telomere length of chromosomes decreases with both *in vitro* and *in vivo* ageing in both of these cell types (Allsopp *et al.*, 1992; Chang and Harley, 1995; Harley, 1991; Harley *et al.*, 1990; Vaziri *et al.*, 1993). In addition, it has been shown that the telomere length in lymphocytes progressively declines as a function of donor age from new-born to great-grandparents in their eighties (Frenck *et al.*, 1998). If telomere length is indeed the mitotic clock, then initial telomere length should give a strong indication of the replicative potential of the cell. Allsopp *et al.* (Allsopp *et al.*, 1992) confirmed this in a study on fibroblasts from a number of donors: they showed a striking correlation, valid over the entire age range of the donors, between replicative capacity and initial telomere length, providing additional support for the telomere hypothesis of cellular ageing.

1.5.4 Telomere Length as a Marker of Disease

If telomeres shorten in an age-dependent manner, and cells enter senescence when telomeres reach a critically shortened length, it follows that an increase in the number of

shorter telomeres, or rapid attrition of telomeres will result in premature senescence, and this may have implications for the development and /or progression of various diseases. Werner's Syndrome displays characteristics of premature ageing, including atherosclerosis (Chakraverty and Hickson, 1999; Salk, 1982). The cause is a defect in the RecQ family of DNA helicases (Mohaghegh *et al.*, 2001a) resulting in accelerated telomere loss (Schulz *et al.*, 1996b) and erratic telomere length distributions (Wyllie *et al.*, 2000). Individuals with Down's Syndrome (trisomy 21) experience premature immuno-senescence, and considerably accelerated telomere loss (130bp/year) has been demonstrated (Vaziri *et al.*, 1993) in the lymphocytes of Down's subjects. Accelerated telomere loss has also been observed in Fanconi anaemia (FA) (Callen *et al.*, 2002; Leteurtre *et al.*, 1999), scleroderma (Artlett *et al.*, 1996), and ataxia telangiectasia (AT) (Metcalfe *et al.*, 1996; Vaziri *et al.*, 1997; Vaziri, 1997), disorders associated with chromosome instability. The number of telomere repeats in individuals with chronic hepatitis and liver cirrhosis has been shown to be lower than in normal liver of the same age (Aikata *et al.*, 2000), and it has been suggested that the fibrotic scarring at the cirrhosis stage is a consequence of hepatic telomere shortening and senescence (Wiemann *et al.*, 2002). Of course, it is possible that shortening simply reflects the inflammatory nature of disease processes. For example, the telomere length of the rectal mucosa of individuals with ulcerative colitis are significantly shorter than controls (Kinouchi *et al.*, 1998; O'Sullivan *et al.*, 2002), leading to increased chromosome instability. Similarly, the $CD8^+$ subset of T-lymphocytes suffer extreme loss in patients with HIV (Batliwalla *et al.*, 2000).

Telomere biology also plays a key role in cancer, as the most important property of malignant neoplasms is that they have the ability to go through countless cycles of replication followed by cell division. This is largely due to the activation of telomerase, usually at the stage when progression from contained lesions to invasive cancer occurs (Shay and Bacchetti, 1997). Indeed, activity has been detected in $\approx$ 70% of stage I breast tumours and > 95% stage I-IV tumours (Hiyama *et al.*, 1996). Surprisingly, given that telomerase adds telomeric repeats, the telomeres of proliferating cancer cells are often quite short (de Lange *et al.*, 1990; Hastie *et al.*, 1990); this may be a result of the telomerase capping the telomere, preventing the cell entering senescence, without actually extending it. Telomere lengthening in cancerous cells has also been observed in the absence of telomerase (Bryan *et al.*, 1997; Dunham *et al.*, 2000; Henson *et al.*,

2002). This is referred to as ALT (Alternative Lengthening of Telomeres), and occurs via homologous recombination and copy switching (Dunham *et al.*, 2000).

Interestingly, there is an increasing amount of evidence that replicative senescence functions as a tumour-suppressor mechanism. Firstly, cells with a finite replicative life span are orders of magnitude less likely to form tumours than immortal cells (Newbold *et al.*, 1982). Secondly, certain oncogenes act, at least in part, by immortalising or extending the life span of cells (Sager, 1991; Shay *et al.*, 1991). And thirdly, among genes essential for establishing and maintaining the senescent phenotype there are two well known tumour suppressors: the p53 and retinoblastoma (Rb) genes (Sager, 1991; Shay *et al.*, 1991). p53 and Rb appear to be essential for cells to irreversibly arrest growth once their telomeres reach a critically short length (a terminal restriction fragment of about 4kb in most somatic cells.

Telomere structure, in addition to length, is a critical factor

While the absolute length of the telomere is important, the review of the role of telomere-binding proteins and the precise configuration of the telomeric DNA suggest that length is not the only important factor. Studies of genes on truncated yeast telomeres have revealed a continuous domain of transcriptional silencing with a strength of effect that is proportional to the distance from the telomere (Renauld *et al.*, 1993). Telomeres regulate chromatin structure in yeast (Chan and Blackburn, 2002), and evidence from studies on human cells now suggests that telomere *structure,* rather than *length* alone, is important in replicative senescence (Rubio *et al.*, 2002). This is supported by evidence that the control of human TPE may not be due to the telomere length alone, but due to accompanied changes in chromatin structure (Koering *et al.*, 2002). It has also been suggested that changes to TPE could influence immortalisation; proteins liberated from shortening telomeres silencing tumour suppressor genes (Wood and Sinclair, 2002). This hypothesis could explain why most telomerase-positive cancer cells retain relatively shorter telomeres.

1.5.5 Telomeres & Ageing of the Cardiovascular System

1.5.5.1 Pulse pressure & pulse wave velocity

Carotid arterial wall thickness, as assessed by intima-media thickness (IMT), increases with age in adult humans (Kornet *et al.*, 1998; Kuller and Sutton-Tyrrell, 1999; O'Leary

et al., 1999), and is an independent risk factor for cardiovascular disease (Bots *et al.*, 1997; Kuller and Sutton-Tyrrell, 1999; O'Leary *et al.*, 1999). Observations from animal studies indicate that sustained elevations in local distending pressure are capable of stimulating smooth muscle hypertrophy and synthesis of extracellular materials in the arterial wall (Leung *et al.*, 1976), and Tanaka *et al.* (Tanaka *et al.*, 2001) have hypothesized that increasing carotid IMT with advancing age in humans may be associated with elevated carotid systolic BP.

Pulse pressure (PP) is influenced by arterial stiffness (Nichols and O'Rourke, 1998), but is also affected by the timing and intensity of wave reflections resulting from the summation of a forward wave coming from the heart and propagating at a given speed (pulse wave velocity, PWV) toward the origin of resistance vessels, and a backward wave reflecting towards the heart from particular sites (Nichols and O'Rourke, 1998). In subjects over the age of 50 PP is an independent marker of cardiovascular risk, particularly for MI (Safar, 2001). Ventricular ejection also tends to be reduced, and arterial stiffness becomes a major determinant of increased systolic BP and PP. Arterial stiffness as measured by PWV has been shown to independently predict cardiovascular risk (Blacher *et al.*, 1999; Laurent *et al.*, 2001).

Altered mechanical properties of the aortic wall influence the level of aortic systolic BP (which is increased), and diastolic BP (which is decreased) as a consequence of early wave reflections. These alterations are influenced by factors such as hypertrophy, or remodelling, of arterial and arteriolar vessels (Mulvany and Aalkjaer, 1990). Age influences all of these modifications and tends to increase PP more rapidly in central than in the distal compartment of the arterial tree. Changes to PP resulting from calcified plaques, particularly at aortic, carotid and femoral bifurcations, and at the origin of renal arteries, may produce reflections sites closer to the heart (Latham *et al.*, 1985; Safar, 2001). This may also modify wave reflections and increase PP.

As PP and PWV both increase with age, an association between telomere length and one, other, or both of these would provide support for the telomere hypothesis of ageing. Two recent studies of the relationship between ageing of the human arterial system and telomeric length (Benetos *et al.*, 2001; Jeanclos *et al.*, 2000) have indeed showed that after adjustment for age, telomere length was inversely correlated with PP and that the relationship was modified by sex. Thus, men with shorter telomeres are more likely to exhibit high PP and PWV (Benetos *et al.*, 2001). From these studies,

Aviv (Aviv, 2001) has suggested that the biological age of persons with relatively high pulse pressures is more advanced than their chronological age would indicate, thus reinforcing the theory that telomere biology is involved in cardiovascular disease.

1.5.5.2 The vascular endothelium

The vascular endothelium has been described as an "active paracrine, endocrine, and autocrine organ that is indispensable for the regulation of vascular tone and maintenance of vascular homeostasis" (Bonetti *et al.*, 2003). Some of the major atheroprotective effects are shown in Table 1.5.

Table 1.5: Atheroprotective Effects of the Healthy Endothelium

Effects
Promotion of vasodilation
Antioxidant effects
Anti-inflammatory effects
Inhibition of leucocyte adhesion and migration
Inhibition of smooth muscle cell proliferation and migration
Inhibition of platelet aggregation and adhesion
Anticoagulant effects
Fibrinolytic effects

* taken from Bonetti *et al.* (Bonetti PO *et al.*, 2003)

Endothelial dysfunction in atherosclerotic epicardial coronary arteries was first described by Ludmer *et al.* (Ludmer *et al.*, 1986) in 1986, and is characterised by a reduction in bio-availability of vasodilators, such as nitric oxide, while endothelium-derived contracting factors are increased (Lerman and Burnett, Jr., 1992). Thus there is impairment of endothelium-dependent vasodilation, coupled with a state of "endothelial activation" which is characterised by a proinflammatory, proliferative, and procoagulatory status that favours atherogenesis (Anderson, 1999). Such changes have been shown to increase with advancing age (Celermajer *et al.*, 1994b; Ishida *et al.*, 2003). Bonetti *et al.* (Bonetti *et al.*, 2003) have suggested that, due to its strategic location and biological properties, the vascular endothelium may provide the "missing link" between any given risk factor and its detrimental vascular effect. Many of the conventional risk factors have also been shown to associate with endothelial dysfunction: for example, endothelial function is impaired by hyperhomocyst(e)inaemia (Stuhlinger *et al.*, 2003) and by increased oxidative stress as a result of hyperlipidaemia,

hypertension, diabetes and smoking (reviewed in Cai and Harrison (Cai and Harrison, 2000)). There is also evidence that the risk to develop endothelial dysfunction increases with the number of risk factors present in an individual (Celermajer *et al.*, 1994a; Vita *et al.*, 1990).

Minamino *et al.* (Minamino *et al.*, 2002) have recently demonstrated a direct link between telomere biology and endothelial cell senescence. They inhibited TRF2 in human aortic endothelial cells (HAECs) to induce senescence and found that the cells showed increased expression of ICAM-1 and decreased expression of eNOS, both changes that have been already been implicated in atherogenesis. Conversely, introduction of the catalytic sub-unit of telomerase extended cell life-span and inhibited the functional change associated with senescence. Further evidence linking telomere biology with vascular biology is provided by the observation that increased shear wall stress results in accelerated telomere attrition (Okuda *et al.*, 2003).

These studies suggest that telomere length and/or integrity is important for normal functioning of the arteries, and telomere-induced senescence may play a crucial role in ageing of the cardiovascular system, as manifest by increasing PP, PWV and endothelial dysfunction. Interestingly, measures of arterial stiffness correlate significantly with those of endothelial function (Nigam *et al.*, 2003). In addition, telomere length in patients with vascular dementia has been shown to be shorter than in controls (von Zglinicki *et al.*, 2000), suggesting that telomere dynamics are involved in the biological ageing of both central and peripheral arteries.

1.6 WORK PRECEDING THIS STUDY

If shorter telomeres induce cellular senescence, and senescent endothelial cells demonstrate atherogenic properties, individuals with shorter telomeres may be at increased risk of developing atherosclerosis. In a previous study carried out within our group the mean terminal restriction fragment (TRF) length of leucocyte DNA was compared between 10 patients with severe triple-vessel coronary artery disease and 20 age and sex-matched controls with normal coronary arteries at angiography (Figure 1.17). This study demonstrated a small, but highly significant difference in leucocyte mean TRF length between cases and controls. In both groups there was a clear, age-related decline in telomere length, in agreement with previous studies (Benetos *et al.*, 2001; Vaziri *et al.*, 1993; Wynn *et al.*, 1998). After adjustments for age and sex, cases

had a mean TRF length 303 base pairs shorter than controls (p=0.002) (Samani *et al.*, 2001). The difference between the two groups (303bp) and the rate of loss (35bp/year) suggests that, in biological terms at least, cases have a telomere length similar to that of controls approximately 8.6 years older. This implies that subjects with advanced coronary atherosclerosis are "biologically" older than age and sex-matched control subjects, at least as far as the telomeres of their circulating WBCs are concerned. Interestingly, the trend lines for the cases and controls appear to be parallel, suggesting that rather than cases having had their telomeres eroded at a faster rate than controls, cases may have actually inherited shorter telomeres. If this hypothesis is true, individuals born with shorter than average telomeres would be at an increased risk of developing CHD. One limitation of the study is that the association has been shown in WBCs but not in other tissues. However, a recent study examining telomere length in coronary endothelial cells of 11 patients with CHD and 22 age and sex matched controls also demonstrated significantly shorter telomeres in the cases compared to controls (Ogami *et al.*, 2004).

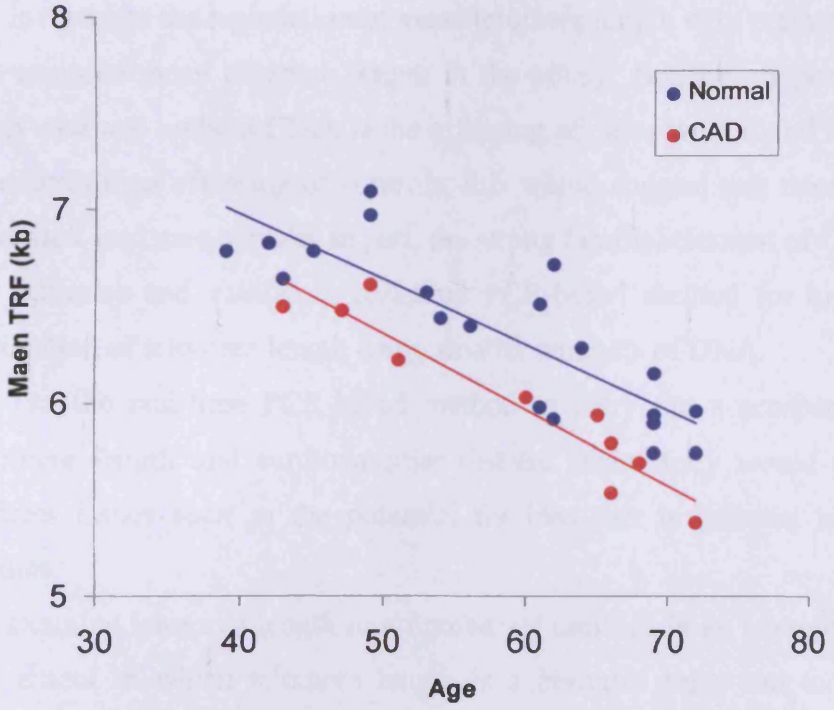


Figure 1.17: Plot of mean length of leucocyte DNA TRFs against age of individuals with severe coronary artery disease or normal coronary arteries (Samani *et al.*, 2001)

1.7 HYPOTHESIS

The incidence of CHD is known to increase with age due to a number of processes including elevated BP and a decrease in the anti-thrombogenic property of the endothelium. There are a number of well-established risk factors, such as hypertension, diabetes and obesity that contribute to the aetiology and pathogenesis of CHD, and much work has been carried out attempting to identify candidate genes involved in CHD. Despite this, there are several aspects that remain unclear, including the highly variable age-of-onset in individuals with very similar risk profiles. The mean telomere length of various cells types has been shown to reduce in an age-related manner, thus telomere length is considered a marker of biological ageing. Chronological ageing is an extrinsic variable that proceeds at the same rate in all individuals of a species, whereas biological ageing is an intrinsic variable that can proceed at different rates in different individuals. Given that telomere length is a marker of biological ageing, and telomere length has been shown to be shorter in certain age-related disorders, we have hypothesised that CHD is a disease of premature *biological* ageing. In order to test this hypothesis we designed a number of studies:

1. To investigate the association of mean telomere length with premature MI.
2. To examine mean telomere length in the young, healthy offspring of subjects both with and without CHD. If the offspring of cases were found to have shorter telomeres than offspring of controls, this would suggest that telomere length is inherited, and may explain, in part, the strong familial element of CHD.
3. To optimise and validate a real-time PCR-based method for high-throughput estimation of telomere length using smaller amounts of DNA.
4. To use the real-time PCR-based method to carry out a prospective study of telomere length and cardiovascular disease. This study would enable me to address issues such as the potential for bias that is inherent in case-control studies.
5. To examine telomere length in a number of families in an attempt to determine the extent to which telomere length is a heritable trait, and to elucidate the genetic basis of telomere length using a genome-wide scan to identify candidate loci.

METHODS

In this section I describe optimisation of the Southern blotting method of telomere analysis, and optimisation and validation of a real-time PCR based method for telomere analysis. Included is a discussion of the advantages/disadvantages of the Southern blotting method, and the potential benefits of using the PCR-based method. More specific methods related to particular aspects of the work are discussed in the relevant chapters.

2.1 DNA EXTRACTION

Blood was drawn into EDTA anti-coagulant tubes and stored at -80°C DNA extractions were carried out using a commercially available kit (PureGene™, Gentra Systems, USA).

2.1.1 Cell Lysis

Frozen bloods were thawed in a 37°C water bath for 30 minutes before 9ml whole blood added to a 50ml tube containing 27ml RBC Lysis Solution. The tube was inverted to mix, and incubated at room temperature on a roller for 10-20 minutes. Centrifugation was carried out in a bench centrifuge (Heraeus Instruments, USA) for 10 minutes at 3100rpm/2000g, and the supernatant poured off into a waste bottle containing disinfectant (Haztab, Flowgen, UK), leaving a visible white pellet and between 300-600µl residual fluid. This was then vortexed vigorously to resuspend the pellet in the residual supernatant.

Cell Lysis Solution (9ml) was then added and the tube inverted several times to lyse the cells. At this time the contents of two tubes (i.e. from the same sample) could be pooled, but all details from here on will assume that this was *not* the case.

If visible clumps formed the tube was incubated for several hours at 37°C, or overnight at room temperature. Alternatively, 45µl Proteinase K (20mg/ml) was added (to a final concentration of 100µg/ml) and incubated at 55°C from one hour to overnight.

At this stage samples are stable at room temperature for 18 months.

2.1.2 RNase Treatment

RNase A (45µl) was added to cell lysate and the sample mixed by inverting the tube 25 times, followed by incubation at 37°C for 15-20 minutes.

2.1.3 Protein Precipitation

Samples were cooled on ice, as this step usually followed the final 55°C incubation during cell lysis. Protein Precipitation Solution (3ml) was added to cell lysate and vortexed vigorously for 20 seconds, left on ice for at least 5 minutes, and centrifuged at 3100rpm for 10 minutes. The precipitated proteins form a dark brown pellet.

2.1.4 DNA Precipitation

The supernatant only (containing the DNA) was poured into a clean 50ml tube containing 9ml 100% isopropanol (propan-2-ol) and the sample mixed by inverting 50 times, until DNA precipitation could be seen. Centrifugation was carried out at 3100rpm for 5 minutes and the supernatant poured off, taking care not to lose the pellet. The tube was then inverted on clean tissue to drain the residual liquid. The pellet was washed by inversion in 9ml 70% ethanol, centrifuged at 3100rpm for 5 minutes and the ethanol poured off. Again the tube was inverted on clean tissue for a maximum of 10 minutes to drain the residual fluid.

2.1.5 DNA Hydration

DNA Hydration Solution (TE, 750µl) was added such that the pellet was in solution and left at 4°C overnight. The sample was mixed and centrifuged briefly at 3100rpm before incubation at 65°C in a water bath for one hour. Following a further mix the sample was then allowed to stand at room temperature overnight. The sample was then mixed and centrifuged at 3100rpm (pulse spin), and incubated for a further hour at 65°C in a water bath. When cool the sample was stored a 4°C until DNA determination was carried out.

2.1.6 DNA Quantification

DNA was quantified in duplicate by spectrophotometry using. Nucleic acids have a peak absorbance in the ultraviolet range, at approximately 260 nm. A solution containing 50µg/ml of double stranded DNA has an absorbance of 1 at 260nm. When the spectrophotometer has a path length of 1 cm, absorbance (A_{260}) = optical density

(OD), where OD = extinction coefficient (E) x concentration. Extinction coefficients vary with the type of nucleic acid: double stranded DNA (dsDNA) has an E = 20 g-1cm-1L. Thus,

$$\text{DNA concentration} = A_{260}/20 \text{ x dilution factor}$$

The 260/280 ratio was included to give an indication of sample purity: pure DNA has an A260/A280 ratio of 1.8-1.9. Lower ratios indicate substantial protein contamination, while a higher ratio generally indicates RNA contamination. Those samples found to be too dilute were ethanol precipitated and re-suspended in a smaller volume.

2.1.7 Assessing DNA Integrity

The integrity of all DNA samples was analysed once the extracted DNA had re-suspended fully and before the sample was used in any experiment.

5ul loading buffer (0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF; 15% (w/v) Ficoll) was added to 2ug of genomic DNA and the solution was loaded on to a 1% (w/v) agarose gel (ICN), made with 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. Both 1kb and 5kb DNA ladders (Invitrogen) were also loaded on to the gel to determine the size of the genomic DNA. Both the gel and the running buffer contained 0.05ul/ml of 10mg/ml ethidium bromide (Sigma). The gel was run at 80 volts in 1 x TAE buffer for roughly 1 hour and was analysed using a UV light box. Significant smearing down the gel (i.e. more than 1-2cm) suggested that the DNA was not of good quality.

2.2 DETERMINATION OF MEAN TELOMERE LENGTH BY TERMINAL RESTRICTION FRAGMENT ANALYSIS

2.2.1 Overview of Terminal Restriction Fragment (TRF) analysis

The basic principle of TRF analysis is illustrated in Figure 2.1. Frequently cutting restriction enzymes (*RsaI,* 5'...G$^{\nabla}$ANTC...3', and HinfI, 5'...GT$^{\nabla}$AC...3') are used to digest genomic DNA; telomeric DNA remains intact, as there are no recognition sites for the enzymes used. The digested DNA is then separated using agarose gel

electrophoresis, with the small, digested fragments rapidly reaching the bottom of the gel. The much larger, undigested telomeric DNA (Figure 2.1, small red boxes) separates at a much slower rate, forming a 12-15cm smear that can then be analysed.

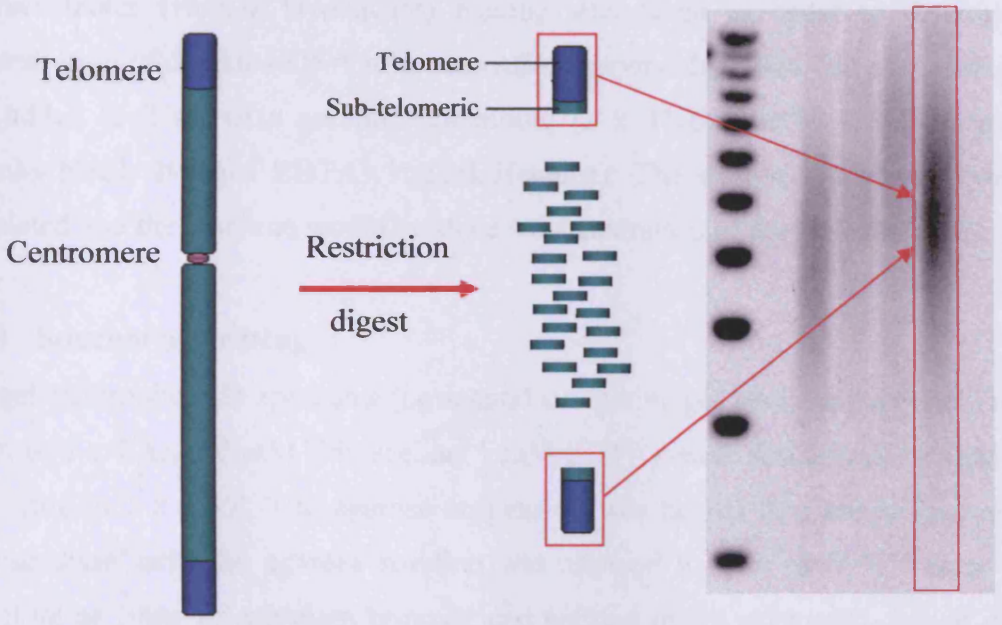


Figure 2.1: Overview of TRF analysis. Genomic DNA is digested, leaving the telomeres intact. Separation on an agarose gels removes the digested fragments, and leaves a telomeric "smear".

The smear is due to inter- and intra-chromosomal, in addition to inter- and intra-cellular differences in telomere length. If every telomere on every chromosome of every cell in the population analysed were the same length then there would be just one, discrete band. It is important to note that a small, variable amount of sub-telomeric DNA remains intact and is captured in the telomeric smear (this has implications for analysis that will be discussed later).

2.2.2 Restriction Enzyme Digest of Genomic DNA

8ug genomic DNA were digested to completion with 15U of *Rsa I* and *Hinf I* (Invitrogen) overnight at 37°C. The digestion always took place in a buffer containing a

final concentration of 1 x One Phor All buffer (OPA) (Amersham Pharmacia), made to the correct volumes with distilled water.

2.2.3 Fluorometry Readings of Digested DNA Samples

A fluorometer (Hoefer Dynaquant) reading was taken in order to determine the concentration of digested DNA after restriction enzyme digestion. 2ul of digested DNA was added to 2 ml of a solution containing (1 x TNE (50mM Tris-HCl (pH 8.0), 150mM NaCl, 100mM EDTA) 1ug/ml Hoechst). The excitation emission was then calculated and the machine would produce a concentration of the DNA in ng/ul.

2.2.4 Southern Blotting

The gel electrophoresis apparatus (horizontal submarine gel tank) was set and a running buffer of 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) used. A 0.5 % (w/v) agarose gel was made in 1 x TAE. The agarose suspension was heated in a microwave until the agarose dissolved. The agarose solution was allowed to cool to 60°C before adding 0.05ul/ml of 10mg/ml ethidium bromide and pouring into a gel mould. 2ug of digested DNA in 6ul of loading buffer (0.25% (w/v) bromophenol blue; 0.25% (w/v) xylene cyanol FF) was loaded onto the gel. Size standards were run on either side of the gel (500ng KB DNA, Invitrogen, and 700ng Hyperladders I and VI, Bioline). The gel was run overnight in the 1 x TAE running buffer with 0.05ul/ml of 10mg/ml ethidium bromide at 4°C. The samples were initially run from the wells at 150V for 1 hour and then the voltage was reduced to 50V for the continuation of the overnight procedure. The gels were examined with the use of an ultraviolet transilluminator (Alpha Innotech Corporations) to ensure that the genomic 'smear' had run to the bottom of the gel and this smear was also used to ensure equal loading of the samples.

Gels were depurinated in 250ml of 0.25HCl (Fisher) (6.25ml/250ml) depurinating solution for 20 minutes with gentle shaking. The solution was changed for fresh after 10 minutes. The usual method of depurinating until the bromophenol blue dye in the loading buffer turns from blue to yellow was not possible as this had usually run off the gel completely. Gels were then briefly rinsed in distilled water and then immersed in a denaturing solution containing 1.5mol/L NaCl, 0.5 mol/L NaOH for 30 minutes with gentle shaking. Gels were rinsed again with distilled water and then washed in a

neutralising solution containing 1.0 mol/L Tris-HCl, 1.5 mol/L NaCl pH 7.2 for 30 minutes, with gentle shaking. Gels were then rinsed for a final time.

DNA was transferred using a standard Southern blotting technique (Southern, 1975); a pre-wet 3MM paper wick was placed on a glass plate, with both ends immersed in a tray of 10X SSC. The processed gel was placed on the wick, two sheets of pre-wet 3MM paper placed on top, followed by 5-10 dry sheets of QuickDraw™ Blotting Paper (Sigma). A second glass plate and a 1kg weight were placed on the top, and transfer allowed to proceed via capillary action for approximately 2 hours. The sheets of QuickDraw in contact with the 3MM paper were changed after approximately 10 minutes.

When the transfer was complete, the Southern blot was dismantled, and the membrane allowed to air-dry for 20 minutes. The DNA was then covalently cross-linked to the membrane by exposure to 7 x 10^4 J/cm^2 of UV light by placing the membrane for 70 seconds on a UV transilluminator. The membrane was stored at 4°C until the hybridisation process was carried out.

2.2.5 Hybridisation

Pre-hybridisation and hybridisation were performed at 42°C in a hybridisation oven (Hybaid). Membranes were rolled up and placed into a hybridisation tube with 12ml RapidHyb™ Buffer solution (Amersham Biosciences). The membrane was pre-hybridised at 42°C for a minimum of 1 hour.

Terminal deoxynucleotidyl transferase (Tdt) labelling of T_1 probe

Tdt labelling was performed using a commercially available kit (Amersham Pharmacia). 20pmol of T_1 telomere probe $(AATCCC)_3$, 1X Tdt buffer (100 mM sodium cacodylate, pH 7.2, 0.2 mM 2-mercaptoethanol, 2 mM $CoCl_2$) 7.5μCi ^{32}P dCTP, 10mM $MgCl_2$ and 50 units Tdt enzyme. The reaction was allowed to proceed for 1 hour at 37°C, followed by 100°C for 8 minutes.

RadPrime labelling of ladders

RadPrimer labelling was performed using a commercially available kit (Invitrogen). A combination of KB DNA ladder (30ng) and Hyperladders I and VI (45ng) in a total volume of 21μl were incubated at 100°C for 10 minutes, and then immediately placed

on ice. The reaction was made up to 50µl with 1x Radprime buffer (50mM Tris-HCl (pH 6.8), 5mM magnesium chloride, 10mM 2-mercaptoethanol, 150µg/ml oligodeoxyribonucleotide primers (random octamers)), 30µM dATP/dGTP/dTTP mix, 12.5µCi ^{32}P dCTP and 40 units Klenow fragment. The reaction was allowed to proceed for 15 minutes at 37°C, followed by 100°C for 10 minutes.

At this point approximately 2ml of the pre-hybridisation solution was poured into a 15ml sterilin tube and both the labelled ladders and the T1 probe added directly. This mix was then poured back into the hybridisation tube, given a gentle shake to mix, and hybridised at 42°C for 2-4 hours. Washing in 5xSSC/0.1% SDS was carried out at 42°C for 10-15minutes; if the counts were still high and indicating high background, an additional and more stringent wash in 3xSSC/0.1% SDS was performed. The membrane was then allowed to dry for approximately 15 minutes before being wrapped in Saran-wrap and exposed to autoradiographic film within an intensifying screen at –80°C. Film was developed in a Kodak photo processor.

Membranes were routinely exposed overnight and if the signal was strong enough to produce even a faint image, the membrane was then transferred to a phosphorimager plate and exposed for a further 24 hours. The plate was then scanned using a Molecular Dynamics Storm Phosphorimager to provide a digitised image of the hybridised membrane.

Molecular Dynamics ImageQuant™ version 3.3 software was used to analyse the images.

Re-hybridising membranes

Occasionally it was necessary to re-hybridise a particular membrane. A container of water (500-1000ml) was heated to 100°C and the radio-labelled membrane was completely immersed. The container was then placed on an orbital shaker, and the water allowed to cool to room temperature, resulting in removal of the probe. The membrane was either re-hybridised immediately, as detailed previously, or stored at 4°C.

2.2.6 Estimation of mean TRF

Once the gel had been exposed to the phosphorimager, the digitised image was opened using Molecular Dynamics ImageQuant 3.3 software and intensity data for each lane obtained. Background signal was estimated by drawing a rectangle object in the space between the ladder lane, and the first sample lane; this allowed an estimate of the "sum above background" to be determined for any region of the gel image.

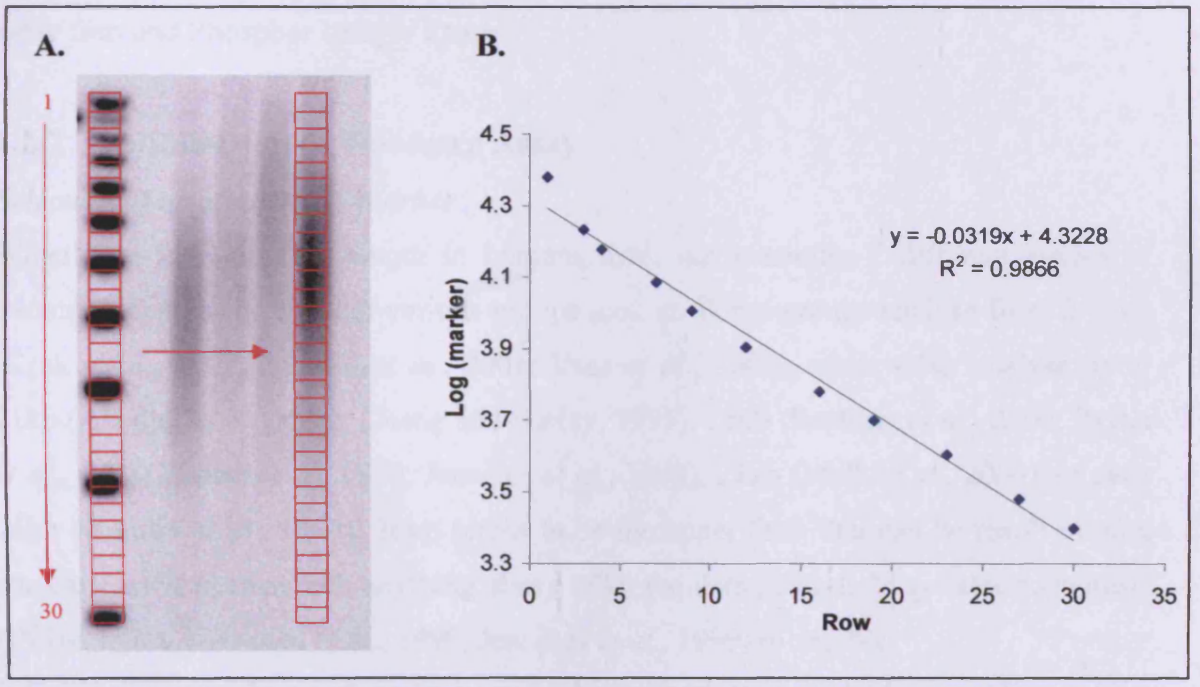


Figure 2.2: Estimating mean TRF. A. Using ImageQuant 3.3 software a grid (1 column x 30 rows) was drawn over the lane containing the KB DNA marker. It was important to try and draw the grid such that each of the bands in the marker lane lies within a row, and the number (1-30) into which each band falls recorded. Where a band lay between 2 rows, it was assigned to that row in which the majority of the band fell. **B.** A graph of log (molecular weight) against row number was plotted and used to interpolate values for each of the rows not containing a band. Thus, an average size (bp) was estimated for each of the 30 regions in the grid.

Figure 2.2 depicts the construction of the standard curve, used to estimate the average molecular weight for each of the 30 regions of the grid. The grid was copied and placed over each sample lane. A rectangle was drawn in a region away from the lanes and defined as 'background', and the image was then quantitated by integrating the volume in each row. The section of output used for downstream analysis was the 'sum above background'. The following equation was then used to estimate the size of the mean telomere restriction fragment (TRF):

$$TRF = \frac{\Sigma OD_i}{\Sigma(OD_i/MWt_i)}$$

where MWt_i is the molecular weight (or size, in base pairs) and OD_i is the optical density (sum above background) at that weight. This formula takes into account the fact that longer telomeres bind more labelled probe and consequently appear darker on the x-ray film and Phosphor Imager image.

2.2.7 Optimisation of Telomere Assay

Selection of a suitable size marker

When studying telomere length in humans there are generally 2 different ranges of telomere lengths (in kb) that various groups look at. Some groups analyse from 2-12kb (Kruk *et al.*, 1995; Samani *et al.*, 2001; Vasa *et al.*, 2000), while other analyse up to 21kb (Allsopp *et al.*, 1992; Chang and Harley, 1995), 23kb (Bestilny *et al.*, 2000; Bryan *et al.*, 1998; Hastie *et al.*, 1990; Jeanclos *et al.*, 1998), 24kb (Melk *et al.*, 2000), or even 26kb (Hultdin *et al.*, 1998). 26kb seems to be the upper limit that can be resolved on a standard 0.5% agarose gel; anything above 26kb requires pulse-field gel-electrophoresis (PFGE) (Golubovskaya *et al.*, 1999; Jennings *et al.*, 1999) to resolve.

In some cases the choice of marker may be due to the system studied i.e. cell lines with a known telomere length, and the commercially available markers at the time. I analysed up to 24kb as that should yield more information for certain smears. Only analysing up to 12kb may result in biased results, as the data above 12kb is lost. The ladders available included 1KB and 5KB DNA ladder (Invitrogen), λ/Hind III fragments (Invitrogen), and Hyperladders I and VI (Bioline), and a comparison is shown in Figure 2.3.

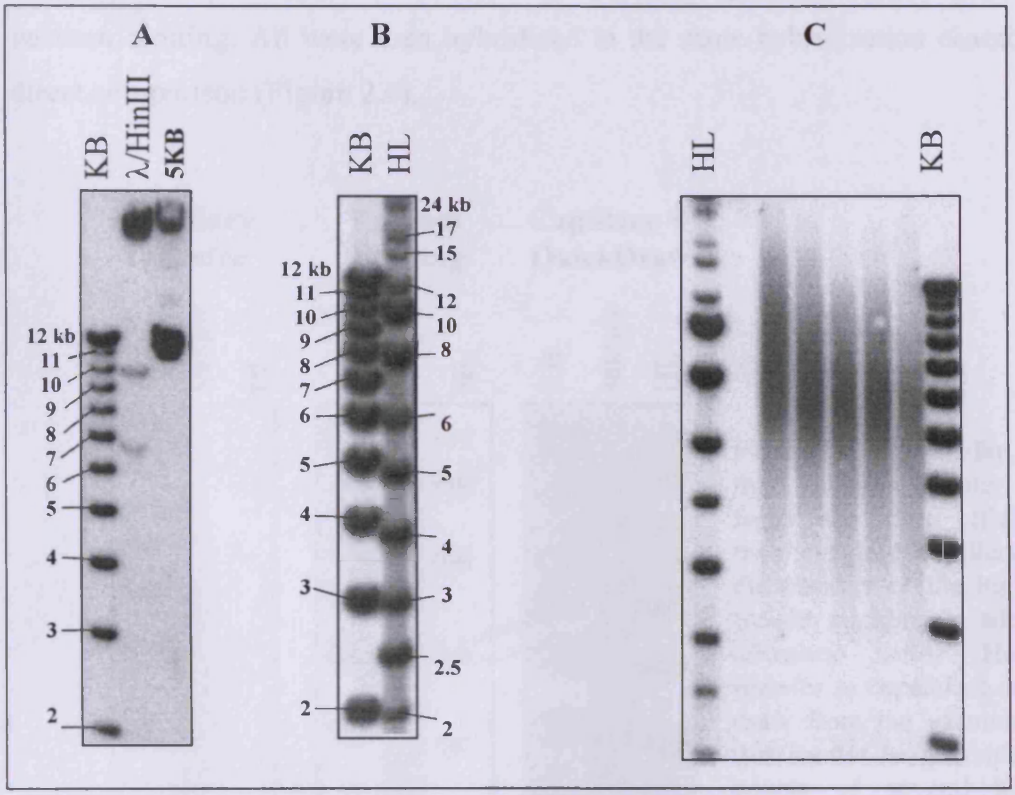


Figure 2.3: Selection of marker. A. The KB ladder was very clear and easy to run, while the 5KB and λ/HinIII ladder did not resolve well on a 0.5% agarose gel (pulse-field would probably be required to obtain adequate separation). **B.** In contrast, the combined hyperladders (HL) were clear and resolve relatively well up to 24kb. Figure **C.** illustrates the problem with using the KB ladder alone; a considerable part of the smear can be present above 12kb (the largest band using KB DNA ladder). This section is not analysed and may lead to bias in the results; the estimated TRF would be lower using KB as opposed to HL. A separate experiment in which a sample was run with both KB ladder and the combined HL's confirmed this prediction: estimated TRF length of sample P089 was 5.7kb using KB, and 6.0kb using HL (data not shown). This error would NOT be the same for all samples; those samples with larger smears (i.e. higher on the gel) would lose more data if KB ladder alone were used.

Capillary action versus vacuum blotting

The traditional method of Southern blotting involves stacking paper towels on top of the gel to draw the buffer up through the Hybond-N via capillary action. This usually involves leaving the DNA to transfer for 12-18 hours (overnight). A commercially available towel (QuickDraw, Sigma) is also available; this claims to transfer completely within 1-2 hours. Finally, vacuum transfer also allows rapid, efficient transfer. To confirm this, identical samples were run on the same gel, the gel was processed as usual before being cut into three with a sterile blade. One third was transferred via standard

capillary action, one third via capillary action using QuickDraw, and the third via vacuum blotting. All were then hybridised in the same hybridisation chamber to allow direct comparison (Figure 2.4).

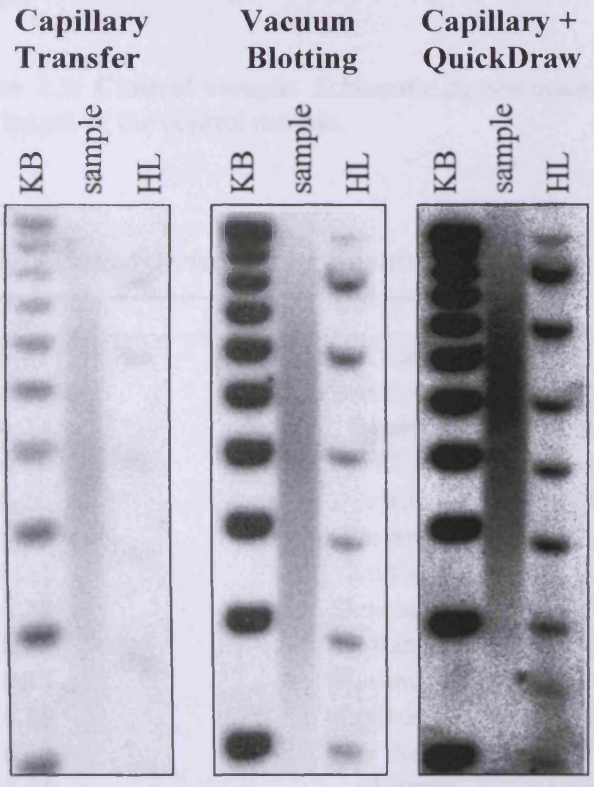


Figure 2.4: Transfer. It is clear that vacuum transfer provides a faster and more efficient method than standard capillary action for the transfer of the high molecular weight markers, in addition to the telomeric smear. However, the transfer is dependent on a constant draw from the vacuum pump, and this cannot be guaranteed over the course of several hours. Using Quickdraw provided the most efficient method, without the need for expensive equipment, or a constant vacuum.

Inter-gel normalisation

Considerable differences in mean TRF estimation can occur between gels (Slagboom *et al.*, 1994). To try and overcome the inter-gel variation, a control sample was included on each gel run, allowing results to be directly compared between gels, and also between studies. A sample was chosen (P089) that had provided a high yield and was digested in seven separate reactions. One aliquot from each of the first five digests, five aliquots from the sixth, and four aliquots from the seventh, were run out on three separate gels to provide an assessment of inter- and intra-gel variation. Figure 2.5 shows a schematic representation of the experiments carried out to obtain an estimate of mean TRF length for the control sample.

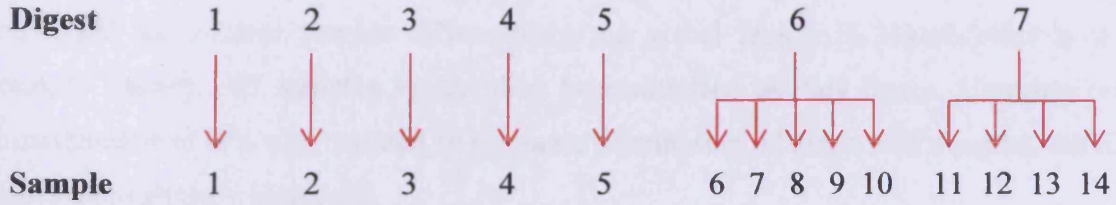


Figure 2.5: Control sample. Schematic representation of digests used to calculate mean TRF length of the control sample.

Table 2.1: Characterisation of a control sample

Gel	*kb*		
1	6.41	Mean	6.337143
1	6.31	Standard Error	0.032924
1	6.44	Median	6.335
1	6.18	Mode	6.18
1	6.26	Standard Deviation	0.12319
2	6.37	Sample Variance	0.015176
2	6.34	Kurtosis	-0.58097
2	6.24	Skewness	0.254388
2	6.17	Range	0.41
2	6.33	Minimum	6.17
3	6.18	Maximum	6.58
3	6.46	Sum	88.72
3	6.58	Count	14
3	6.45	Confidence Level(95.0%)	0.071128

Table 2.1 summarises the results for the control sample: sample P089 was estimated to have a mean TRF of 6.34 ± 0.12kb and was deemed a suitable control sample. The sample was then added to the last lane of each gel run and if, after analysis, the mean TRF was estimated to be within 2 standard deviations of the mean (6.09-6.58kb) the gel was considered suitable to be included in the study. Where the control sample was outside 2SDs, the gel was repeated.

After analysis the mean TRF of the control sample was compared to the mean. The difference was calculated, and all samples were adjusted by the same value. For example, if the mean TRF of the control sample was found to be 6.24kb, all samples on the gel would have their mean TRF's increased by 0.1kb (the difference between the average, 6.34kb, and the value from that particular gel, 6.24kb). Normalisation can also

be performed by determining the factor by which the recorded estimate of mean TRF length for the control sample differs from the actual length (6.34kb/6.24kb in the example above). All samples would then be multiplied by this factor. Carrying out normalisation in this way resulted in the same distribution of mean TRF lengths, but all values were slightly increased.

The effect of varying signal strength

A portion of the variability between gels may be a result of variation in the ^{32}P labelling of the telomere probe, or the quality of the hybridisation. In theory this could mean that when an individual filter is probed two or more times, the strength of the signal may vary according to the efficiency of incorporation during labelling and/or the hybridisation. What effect would this then have on the calculated TRF for that sample, as varying signal strength will obviously significantly alter the OD values?

In an attempt to answer this question I have made an assumption: if the signal strength of a sample is different when re-probed, the percentage difference at any point in the smear is the same as at any other point i.e. if the intensity is 50% increased, it is 50% increased at *every* point down the smear. With this assumption in mind it is possible to consider the effect of altering the intensity. The raw data for sample P001 was used; the output from ImageQuant gives a value for 'sum above background' for each row during analysis. I have taken these values and altered them by a certain percentage, and used the new intensity figures to calculate a new value for TRF.

The results are shown in the Table 2.2, below.

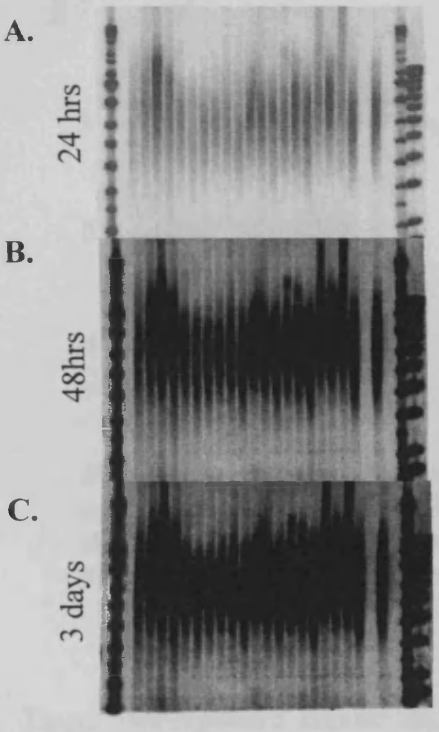
Table 2.2: The effect of altered signal strength:

Row No	*Marker (bp)*	*100% OD*	*OD/MWt*	*120% OD*	*OD/MWt*	*80% OD*	*OD/MWt*	*60% OD*	*OD/MWt*
1	**23990**	1449	0.06	1738.8	0.07	1159.2	0.05	869.4	0.04
2	17378	2018	0.12	2421.6	0.14	1614.4	0.09	1210.8	0.07
3	**17050**	1976	0.12	2371.2	0.14	1580.8	0.09	1185.6	0.07
4	**15000**	2281	0.15	2737.2	0.18	1824.8	0.12	1368.6	0.09
5	13490	5063	0.38	6075.6	0.45	4050.4	0.30	3037.8	0.23
6	**12140**	3098	0.26	3717.6	0.31	2478.4	0.20	1858.8	0.15
7	**10090**	2880	0.29	3456	0.34	2304	0.23	1728	0.17
8	10715	3877	0.36	4652.4	0.43	3101.6	0.29	2326.2	0.22
9	9772	5238	0.54	6285.6	0.64	4190.4	0.43	3142.8	0.32
10	**8000**	6440	0.81	7728	0.97	5152	0.64	3864	0.48
11	8318	11700	1.41	14040	1.69	9360	1.13	7020	0.84
12	7762	15249	1.96	18298.8	2.36	12199.2	1.57	9149.4	1.18
13	**6000**	15144	2.52	18172.8	3.03	12115.2	2.02	9086.4	1.51
14	6607	18440	2.79	22128	3.35	14752	2.23	11064	1.67
15	6026	10366	1.72	12439.2	2.06	8292.8	1.38	6219.6	1.03
16	**5000**	9130	1.83	10956	2.19	7304	1.46	5478	1.10
17	5129	7418	1.45	8901.6	1.74	5934.4	1.16	4450.8	0.87
18	4677	6056	1.29	7267.2	1.55	4844.8	1.04	3633.6	0.78
19	4365	5127	1.17	6152.4	1.41	4101.6	0.94	3076.2	0.70
20	**4000**	5411	1.35	6493.2	1.62	4328.8	1.08	3246.6	0.81
21	3715	3687	0.99	4424.4	1.19	2949.6	0.79	2212.2	0.60
22	3467	2955	0.85	3546	1.02	2364	0.68	1773	0.51
23	3162	3099	0.98	3718.8	1.18	2479.2	0.78	1859.4	0.59
24	**3000**	1771	0.59	2125.2	0.71	1416.8	0.47	1062.6	0.35
25	2754	1772	0.64	2126.4	0.77	1417.6	0.51	1063.2	0.39
26	2512	1808	0.72	2169.6	0.86	1446.4	0.58	1084.8	0.43
27	**2500**	1453	0.58	1743.6	0.70	1162.4	0.46	871.8	0.35
28	2138	1126	0.53	1351.2	0.63	900.8	0.42	675.6	0.32
29	1905	1900	1.00	2280	1.20	1520	0.80	1140	0.60
30	**2000**	1615	0.81	1938	0.97	1292	0.65	969	0.48
Sum	OD	159547		191456		127638		95728	
Sum	OD/MWt	28.2552		33.9062		22.6041		16.953	
TRF	(bp)	5646.65		5646.65		5646.65		5646.6	
TRF	(KB)	**5.6**		**5.6**		**5.6**		**5.6**	

This clearly shows that variations in signal strength do not alter the calculated TRF value, provided the *difference is uniform for the entire smear.*

The effect of exposure time

Using a phosphorimager plate allowed a digitised image of the hybridised gel to be obtained in a relatively short time, typically 12-24 hours. Occasionally it was necessary to leave the gel on the plate for a longer time in order to achieve sufficient intensity, but care was taken to avoid saturation of the phosphorimager plate, as this could result in a black image down the entire length of the smear. Consequently it was impossible to estimate the mean TRF from such an image. Thus I decided to investigate the effect of exposure time by randomly selecting a gel and "over-exposing" it, such that a much darker image was obtained. The data was then analysed and the estimated TRFs compared with the original results (see Figure 2.6).



D.

Sample	*24hrs*	*48hrs*	*3 days*
1	6.13 kb	6.24	6.35
2	6.87	7.02	8.45
3	7.48	7.38	8.49
4	6.75	6.64	7.48
5	6.24	6.21	6.01
6	5.97	6.05	6.12
7	6.06	6.12	6.15
8	5.84	5.89	5.79
9	6.13	6.09	5.94
10	5.41	5.52	5.02
11	6.47	6.61	6.98
12	6.39	6.5	7.24
13	6.31	6.25	6.12
14	6.24	6.31	6.47
15	6.62	6.52	6.74
16	5.84	5.81	5.59
17	8.05	8.28	9.87
18	6.78	6.84	7.24
19	7.49	8.15	9.49
20	6.32	6.35	6.49
P089	6.34	6.34	6.34

Figure 2.6: Increasing exposure time. The gel was exposed for 24hrs (**A**), 48hrs (**B**) and 72hrs (**C**). The image for each time point was analysed and normalised to give TRF lengths for all samples (**D**). Results were compared to investigate the effect of overexposure.

It was clear that the exposure time played an important role in determining the final value; the difference from 24-48hrs was relatively small, but 72-hour exposure resulted in significantly different values (see Figure 2.7 below), some of which seemed

unrealistic (samples highlighted by arrows: 2, 3, 17 and 19 at 72 hours). As one would expect, the more overexposed the lane was, the more erratic the results.

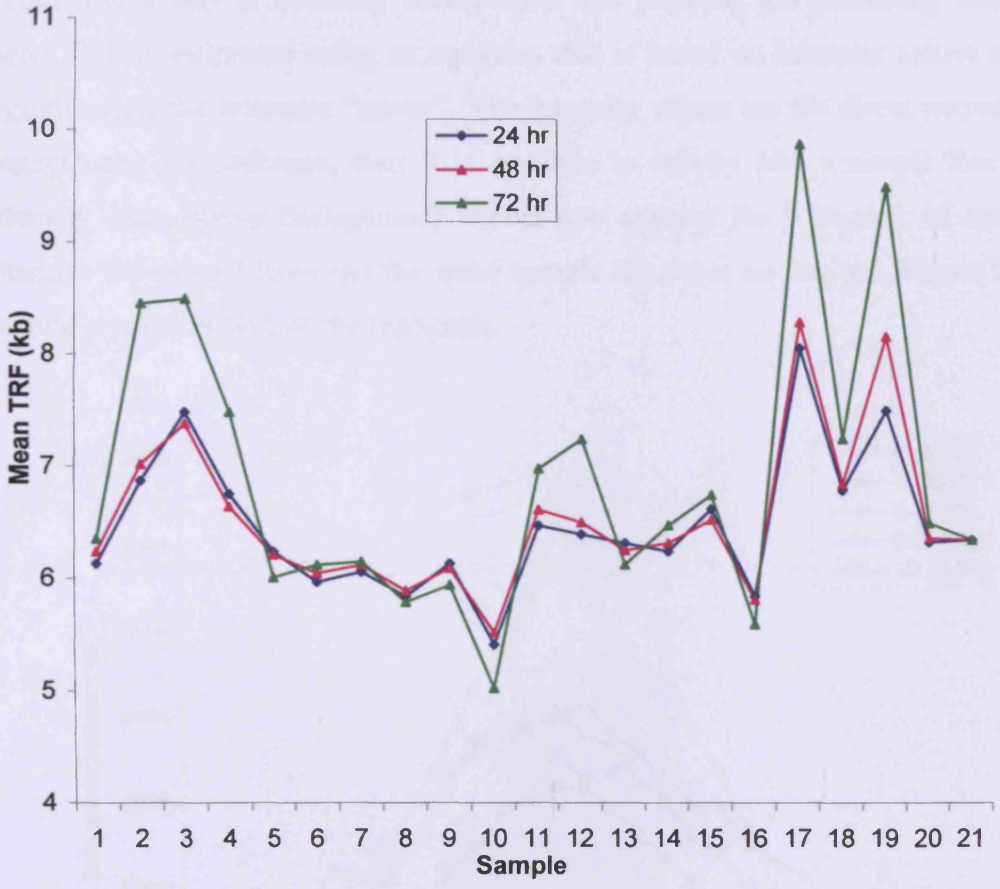


Figure 2.7: Effect of increased exposure time. The mean TRF's for all samples at each exposure time are shown; exposing for 48hrs lead to slightly differing mean TRF's, while 72-hour exposure resulted in markedly differing results for some samples.

Thus, overexposure should be avoided; a brief 18-24 hour exposure will usually be sufficient and if the image is too faint it can be re-exposed. However, it is important to realise that each gel will have an "optimal" exposure time, due to factors such as age of radiolabel, incorporation efficiency of radiolabel, efficiency of hybridisation and stringency of washing.

Artefacts on the blot

Even after washing of hybond-N membranes following hybridisation there can still be "spots" or "smears" present on the autoradiogram. Such artefacts can lead to spurious results and a way of avoiding such results was required. As previously outlined, the mean TRF is estimated using an equation that is based on intensity values at discrete regions along the telomere "smear". The intensity values are the direct output from the ImageQuant 3.3 Software, thus it is possible to rapidly plot a simple line graph of intensity (sum above background) versus row number for a number of samples. To illustrate the point I have run the same sample ten times on one gel. Figure 2.8 shows the line graphs for five of the replicates.

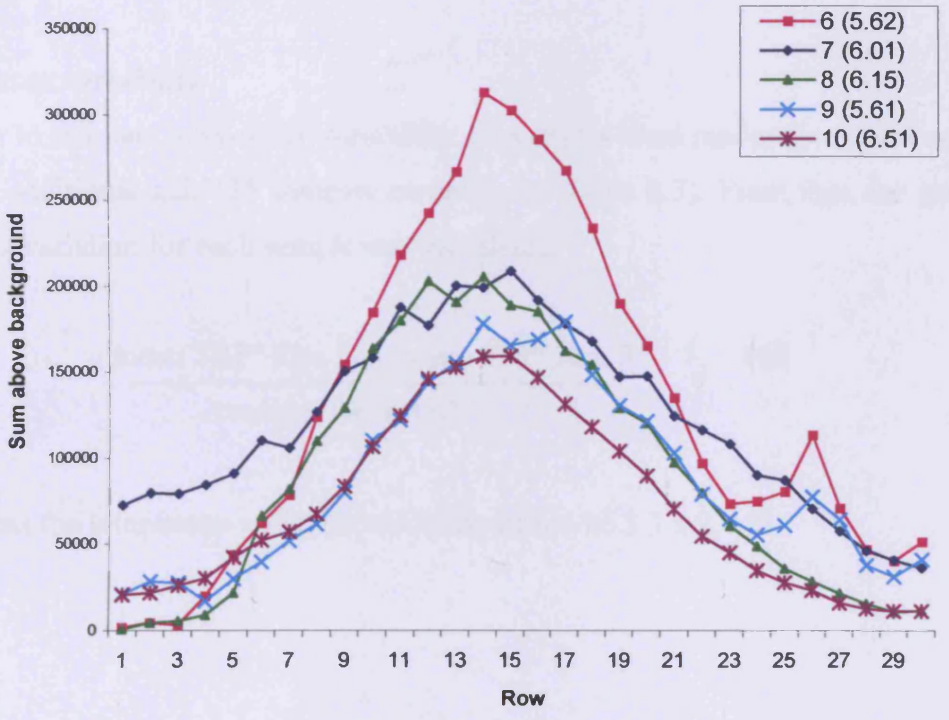


Figure 2.8: Line graph of intensity v row. The intensity (sum above background) is plotted against row number, with row 1 being the top of the smear (approx 24kb), and row 30 being at the bottom (2.5kb). The legend shows the sample number along with the estimated mean TRF (kb) in brackets.

The small peaks at row 26 in replicates 6 and 9 are due to the presence of artefacts on the blot, possibly due to incomplete washing prior to exposure to either the phosphorimager plate, of autoradiographic film.

The effect these peaks can have on the estimated mean TRF is considerable; replicates 6 and 9 give considerably lower TRF values compared to the other replicates. If this were to occur in the control sample lane, the adjustments for inter-assay variation would be affected. Thus, I decided that if a second peak was seen on the profile of a specific sample, and the presence of a spot or smear was evident from the autoradiograph, I would attempt to "smooth" the peak by calculating an average figure from the values either side of the peak. This was not an ideal solution, and perhaps a simple mathematical algorithm would have been more efficient. However, when a clear spot was visible on the film, it seemed reasonable to attempt to ignore the effect on signal, and subsequent estimation of TRF. Occasionally the number of artefacts present on a particular blot was too great, and the samples would be re-run.

Inter-assay variability

In order to estimate inter-assay variability, 50 samples were randomly chosen and re-run on two additional gels (25 samples on each, see Table 2.3). From this, the percentage inter-gel variation for each sample was calculated:

$$\frac{\text{'mean TRF' Run 1} - \text{'mean TRF' Run 2}}{\text{Average 'mean TRF'}} * 100$$

From this the inter-assay variation was estimated to be 3.3 ± 2.7 %

Table 2.3: Inter-assay variability

Sample	mean TRF Run 1	mean TRF Run 2	Ave	% Inter-gel Variation	Sample	mean TRF Run 1	mean TRF Run 2	Ave	% Inter-gel Variation
P001	5.80	6.26	6.03	7.63	P107	8.06	7.85	7.96	2.64
P002	6.73	6.60	6.67	1.95	P115	8.49	8.29	8.39	1.79
P005	6.24	6.53	6.39	4.54	P117	8.67	8.29	8.48	4.48
P011	6.48	6.69	6.59	3.19	P120	8.04	8.23	8.14	2.34
P017	7.24	7.16	7.20	0.28	P123	7.58	7.39	7.49	1.48
P018	6.03	6.30	6.17	1.32	P126	8.39	8.14	8.27	3.02
P019	5.65	5.97	5.81	2.62	P128	6.92	6.86	6.89	0.87
P020	6.57	3.27	4.92	3.88	P129	8.28	7.96	8.12	3.94
P025	7.56	7.41	7.49	2.00	P132	6.37	6.89	6.63	7.84
P030	6.68	6.59	6.64	1.36	P135	7.65	7.89	7.77	3.09
P034	6.34	6.88	6.61	8.17	P142	6.59	6.25	6.42	4.82
P037	6.78	7.09	6.94	4.47	P144	6.16	6.26	6.21	1.61
P038	5.98	6.41	6.20	6.00	P150	8.31	8.44	8.38	1.55
P039	6.58	7.20	6.89	9.00	P160	6.30	6.48	6.39	1.73
P040	5.67	6.01	5.84	5.82	P168	6.60	6.51	6.56	1.37
P045	8.47	8.21	8.34	3.12	P174	7.25	7.09	7.17	1.12
P050	8.26	8.30	8.28	0.48	P176	6.65	6.87	6.76	3.25
P052	7.16	6.98	7.07	2.55	P178	7.89	7.65	7.77	3.09
P066	7.18	7.39	7.29	1.93	P179	7.88	8.12	8.00	3.00
P067	7.08	6.89	6.99	2.72	P183	7.47	7.63	7.55	2.12
P069	6.39	7.14	6.77	11.09	P532	6.57	6.97	6.77	0.43
P076	7.38	7.05	7.22	2.52	P533	6.34	6.59	6.47	1.25
P085	7.69	7.98	7.84	2.03	P536	7.36	6.48	6.92	11.49
P097	6.96	6.98	6.97	0.00	P541	7.75	7.92	7.84	1.79
P106	7.70	7.58	7.64	1.57	P545	8.57	8.15	8.36	2.30

2.3 QUANTITATIVE REAL-TIME PCR ANALYSIS OF TELOMERE LENGTH

Southern blotting to estimate the mean terminal restriction fragment (TRF) length is a robust method that is regarded as the "Gold Standard" of telomere analysis; however, it is subject to some very valid criticism. Setting up and running a standard southern blot can take several hours; the gel itself has to run for approximately 18 hours to achieve adequate separation. The use of QuickDraw™ (Sigma) blotting paper does mean that that transfer can be achieved in a few hours compared to overnight (if using paper towels), but the gel still has to be processed prior to transfer, and this adds another 2 hours to the protocol. Only then can the DNA be hybridised (overnight), exposed (8-72hr) and analysed.

Another major drawback of TRF analysis is the amount of starting material required: 2μg digested DNA are usually loaded onto the gel. This is not a concern when DNA is readily available, such as leucocyte DNA obtained from peripheral blood, but if one wishes to study specific cell types then it may prove difficult, if not impossible, to procure sufficient material.

The final major criticism is that TRF analysis tells us very little about any particular telomere, rather it simply gives an indication of the mean telomere length of that particular sample. Different telomeres within the same cell, and the same telomere within different cells may all have different lengths, and this results in the characteristic telomere "smear". In addition, the smear can also include a variable region of sub-telomeric DNA that contributes to the estimate of mean telomere length.

PCR-based methods for the study of telomeres are generally used when a specific telomere is the target (Baird *et al.*, 2003), but such methods are often complex, and until recently no method for a more global estimate of telomere length using PCR had been available. However, a PCR-based method for estimating the *average* telomere length has recently been developed by Cawthon (Cawthon, 2002); his strategy for estimating relative telomere lengths by quantitative real-time PCR is to measure the ratio of telomere repeat copy number to single copy gene copy number. This ratio should be proportional to the average telomere length. The single copy gene used is the 36B4 gene, which encodes acidic ribosomal phosphoprotein PO, located on chromosome 12

(Boulay *et al.*, 1999). Before describing the method in detail, I briefly introduce the principles of quantitative PCR, below.

2.3.1 The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) takes advantage of the self-replicating nature of DNA, allowing short primers to be used to initiate synthesis of a target sequence by a DNA polymerise (Figure 2.9).

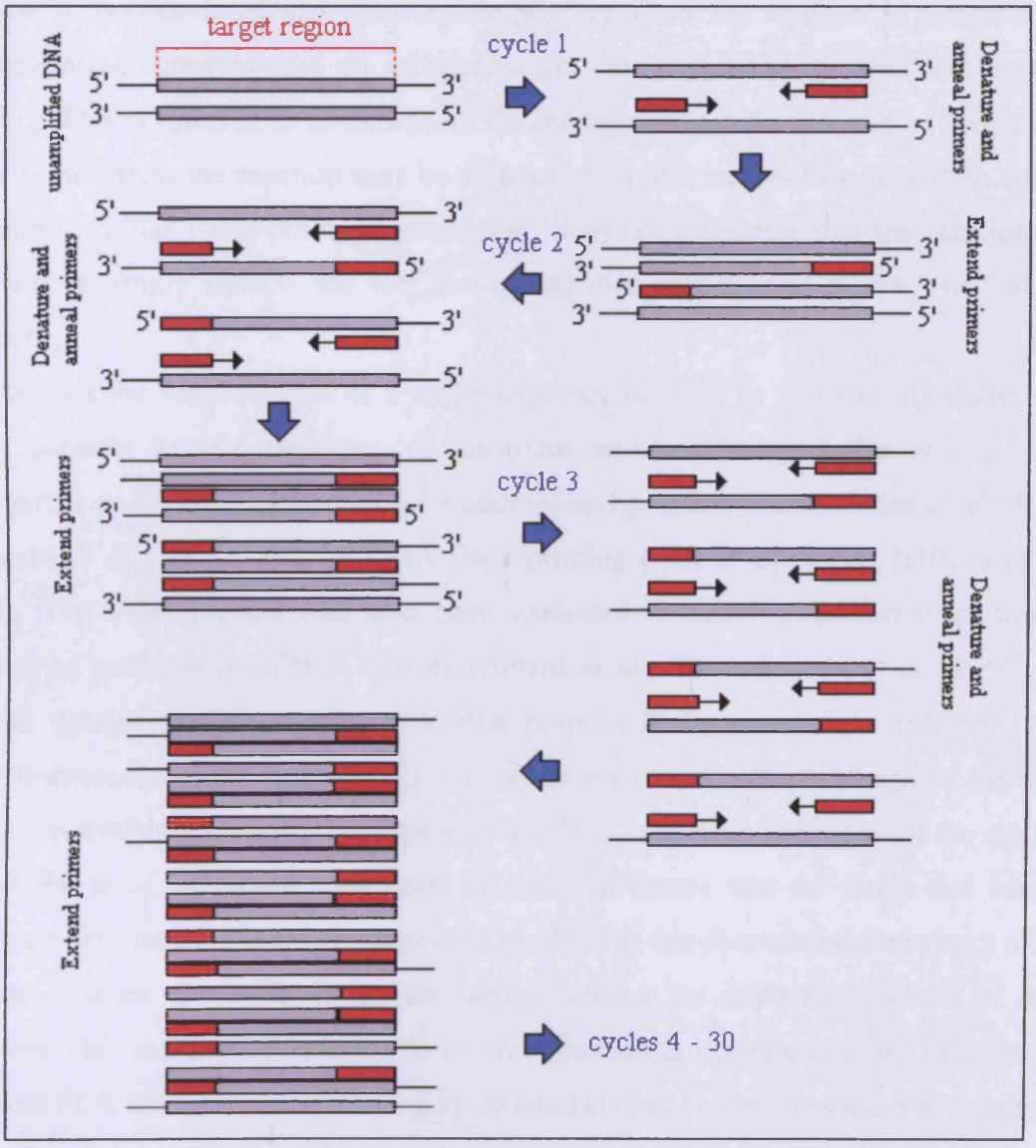


Figure 2.9: PCR. DNA is heated to separate the parent strands, short primers are annealed and extended by DNA polymerase. The process is then repeated (20-40 cycles), resulting in the amplification of the original target sequence.

Theoretically, each round of replication results in a doubling of the target sequence. In practice this exponential phase of amplification only occurs for a limited number of cycles during which reaction components are still in excess and the PCR products are accumulating at a constant rate. While the number of cycles of PCR used can vary, it is unusual to see a protocol using more than 40. This is for a number of reasons, including saturation of amplified target and the eventual failure of the polymerase. At this point the amplification has reached a plateau; additional cycles at this point only result in an increase in primer-dimer and non-specific product. Once the reaction is complete the products are electrophoresed on an agarose gel, and visualised by ethidium bromide staining. This is referred to as **end-point determination**, a term that can be misleading as, in some cases, the reaction may be stopped at a point well before saturation occurs (i.e. simply using "end-point determination" does not guarantee that the reaction has plateued, it simply reflects the fact that quantitation in achieved at the "end" of the reaction).

PCR allows the amplification of a target sequence in order to facilitate its study; it is used routinely for the detection of infectious agents (Rodriguez-Pla *et al.*, 2004; Theegarten *et al.*, 2004), detection of mutations and polymorphisms (Duan *et al.*, 2004; Mabuchi *et al.*, 2004), and for DNA fingerprinting (Gill *et al.*, 1985; Jeffreys *et al.*, 1988). The PCR method has also been extended to allow *quantitation* of targets sequences, particularly mRNA species (Adlard *et al.*, 2004; Lintula *et al.*, 2004), by reverse transcribing the mRNA to cDNA prior to PCR, a technique referred to as reverse-transcription PCR (RT-PCR). The target sequence is compared against a control (a constitutively expressed gene, typically GAPDH) to give an indication of the relative expression level. However, care must be taken to ensure that the target and control genes are not amplified until the plateau is reached, as this decreases the accuracy of the method. Rather, the PCR should be stopped during the exponential phase of each reaction. This requirement can result in time-consuming optimisation, as a number of different PCR reactions using varying cycle numbers need to be carried out to determine when the exponential phase is occurring. This has been overcome with the advent of ***real-time*** PCR, in which the amplification of the target is monitored during every cycle through the use of various fluorescence-based chemistries. This has led to the development of real-time RT-PCR assays (Kubar *et al.*, 2004; Tewari *et al.*, 2004).

2.3.2 Principles of Real-Time PCR

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (in real time, see Figure 2.10). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value indicates the detection of accumulated PCR product.

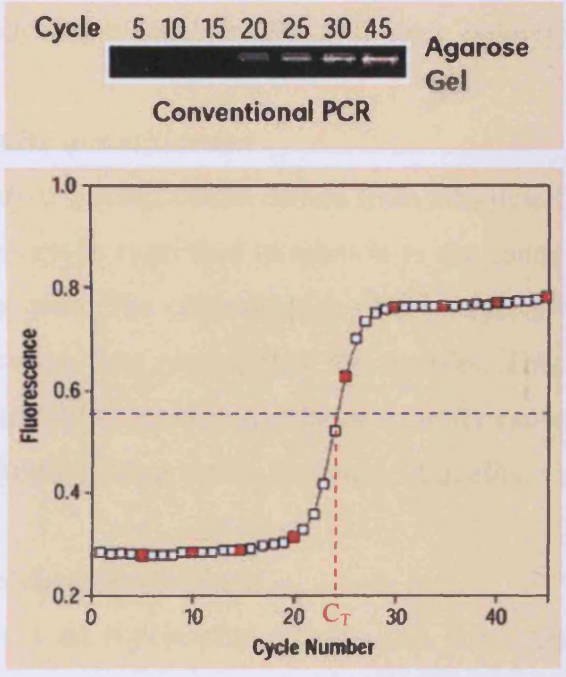


Image adapted from Roche Lightcycler Homepage (http://www.lightcycler-online.com/).

Figure 2.10: PCR quantitation.
A. End point determination. In conventional PCR the target is not visualised until the end of the PCR reaction; each band shown here is from an identical PCR reaction stopped at a different cycle. The intensity of the band at the final cycle provides little information regarding starting template amount.

B. Real-time determination. Data is acquired at the end of each cycle (red points correspond to bands in **A**). Amplification is described as:

$$N = N_0 \times E_{const.}^{\ n}$$

Where N_0 = initial number of molecules; E = amplification efficiency; n = number of cycles. Real-time PCR allows the accurate quantitation of the amount of starting material.

A fixed fluorescence threshold is set (Figure 2.10 – blue broken line) significantly above the baseline, and the parameter C_T (threshold cycle, red broken line) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold (the threshold is the same for all samples within a run). Thus, by monitoring the amount of product at each cycle, real-time PCR allows the user to accurately determine the log-linear phase, and more accurately estimate the starting amount. Achieving this in conventional PCR would require an aliquot of amplified DNA to be removed from the

reaction at each cycle, and run out on an agarose gel. This would result in a profile similar to that shown in Figure 5.2A, but in practice would be very difficult to carry out. When considering real-time PCR there are two basic types of quantification methods available, **absolute quantification** and **relative quantification**.

Absolute quantification

The concentration of the target is expressed as an absolute value that is determined by use of a conventional standard curve, constructed using a serial dilution of a DNA sample of known concentration. The DNA used as the standard is homologous to the unknown samples, and is amplified during the same run. In order for the method to be valid the amplification efficiencies of both the standard and the samples should be identical (see *Amplification Efficiency*, below).

Relative quantification

Relative Quantification differs from Absolute Quantification in that the concentration of the target is expressed in relation to the concentration of a reference gene i.e. a single-copy gene. The concentration of the target is then divided by the concentration of the reference, thus normalising the samples. This normalisation step corrects the samples for any differences in quality or quantity caused by variations in initial sample amount, loading/pipetting errors, nucleic acid quality, variations in PCR efficiency etc.

Amplification efficiency

PCR is an exponential process and, theoretically, the amount of target DNA should double with every cycle of PCR (100% efficiency). In reality the efficiency of the reaction will probably be less than 100%, meaning that we do NOT see a doubling of target with every cycle. The main influences on amplification efficiency are length of the fragment, purity of the sample, the sequence amplified and the PCR primers. The efficiency of any PCR reaction can be determined by generating a standard curve using a serial dilution of the target; the slope of the standard curve can then be converted to amplification efficiency by the algorithm:

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

Inter-assay variability

As with any assay, there will be a degree of inter-assay variability. Correction for such variation in Relative Quantification can be achieved through the use of a "calibrator": a sample that is included in each and every PCR run. Theoretically, if all other factors are constant, the quantification of the calibrator should always give the same result. Results are expressed as the target/reference ratio of the unknown sample normalised by the target/reference ratio of the **calibrator**.

2.3.3 Real-Time Telomere PCR Analysis

Amplifying the telomere using PCR has always presented a problem due to the repetitive nature of the telomere repeats. Software such as PrimerExpress (used to design primers and probes) looks for a unique sequence for each primer site, thus the software fails to suggest possible primer designs.

In 2002 Cawthon published a novel method for estimating telomere length using real-time PCR, and *relative* quantification (Cawthon, 2002). His strategy for determining relative telomere lengths was to measure, for each DNA sample, the factor by which the sample differed from a reference DNA sample in its ratio of telomere repeat copy number to single gene copy number. The principle underlying the telomere PCR is relatively simple: longer telomeres have more potential primer-annealing sites, thus there will be an increase in fluorescence, and a decrease in the number of cycles needed to reach a given threshold, compared to a shorter telomere. The single-copy gene provides sample-to-sample normalisation. The result is then expressed as a ratio, and normalised by using a calibrator sample in every run. The interesting element of the method is the design of the primers for the telomere PCR (Figure 2.11).

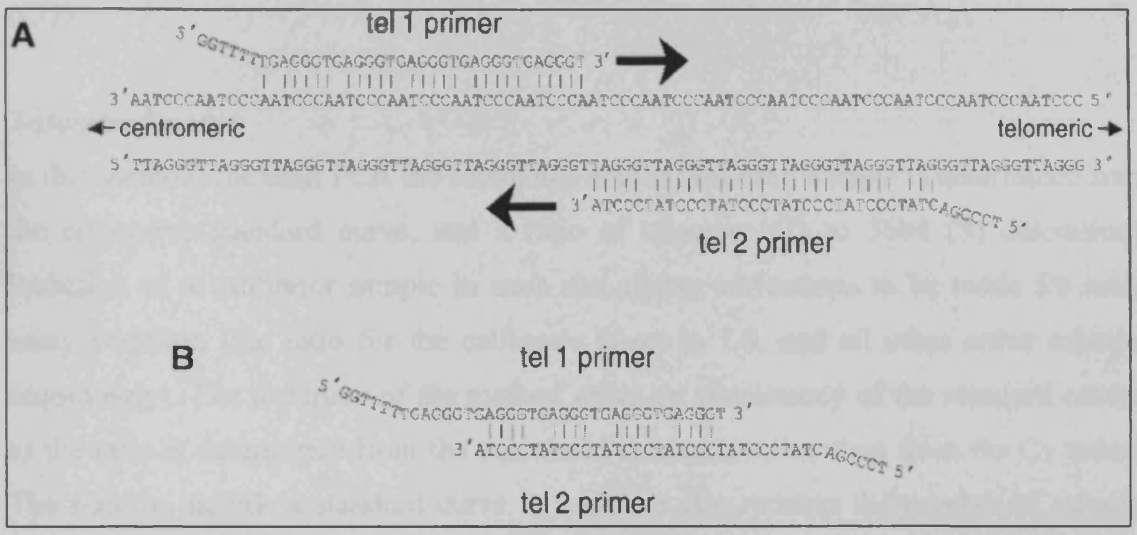


Figure 2.11: Primer design. A. Annealing of primers to genomic DNA. **B.** Annealing of primers to each other. (Adapted from Cawthon, 2002).

Both primers are designed such that every sixth base is mismatched and the last five bases at the 3'-end of the annealed primers are perfectly matched to the template. This provides the substrate for extension by the DNA polymerase. The lower image (Figure 2.11B) shows annealing of primers to each other; the strongest possible hybridisations of the primers to each other involve a repeated pattern of six bases containing four consecutive paired bases followed by two mismatched bases. The 3'-terminal base of each primer cannot form a stable base pair, thereby preventing DNA polymerase from adding additional bases. This minimises the formation of primer-dimer, a problem than can significantly affect Sybr Green-based assays.

It is important to note that products of the same length as telomeres (i.e. 4 -14kb) are not expected. Cawthon proposes that the shortest possible product (76bp, the sum of the lengths of the two primers) is expected at a copy number proportional to the number of primer-binding sites in cycle 1, and, therefore, proportional to telomere length.

Method of analysis

Having run the pair of PCRs (telomere and 36b4), a choice then has to be made regarding the precise method of analysis. Common methods for relative quantification include the 2-standard curves method and the comparative C_T, (or $\Delta\Delta C_T$) method,

however, there are also two more recent methods of analysis that are proposed to increase the accuracy of the assay: Pfaffl (Pfaffl, 2001) and Comparative Quantification (CQ).

2-standard curves

In this method, for each PCR the calculated amount for each sample is determined from the respective standard curve, and a ratio of telomere (T) to 36b4 (S) determined. Inclusion of a calibrator sample in each run allows corrections to be made for inter-assay variation (the ratio for the calibrator is set to 1.0, and all other ratios adjusted accordingly). The accuracy of the method relies on consistency of the standard curves, as the ratio is determined from the calculated amounts, rather than from the C_T values. The need to include a standard curve in each run also reduces the number of samples analysed per run.

Comparative C_T ($\Delta\Delta C_T$)

ABI recommend the comparative ($\Delta\Delta C_T$) method, which assumes ideal amplification efficiency with a doubling of product every cycle, thus the fold change can be calculated from the formula $2^{-\Delta\Delta Ct}$ (Livak, 1997; Livak and Schmittgen, 2001).

Example of analysis

1. calculate the mean C_T values for the calibrator, and unknowns, in both PCRs
2. ΔC_T = mean $C_T^{(telomere)}$ – mean $C_T^{(36b4)}$
3. $\Delta\Delta C_T = \Delta C_T^{(calibrator)} - \Delta C_T^{(unknown)}$
4. Relative Quantification (RQ) Ratio = $2^{-\Delta\Delta Ct}$

The primary advantage of this method is that standard curves only need to be run once, to confirm that the reactions are of equal efficiency. This allows more unknowns to be analysed per run. The negative aspects include the assumptions that 1) the efficiency of both reactions is 2, and 2) the standard curves (and therefore the calculated efficiencies) remain constant.

Pfaffl

In 2001, Pfaffl (Pfaffl, 2001) published a method of analysis for relative quantification that takes into account the relative efficiencies of the two PCR reactions (in this case, the 36b4 and telomere PCR reactions). For each PCR a standard curve is generated, and used to determine the efficiency of the reaction (as detailed previously). The relative efficiencies do not have to be equal, but are incorporated into the analysis, resulting in increased accuracy.

Example of analysis

1. mean C_T values are determined for calibrator and samples for both PCR's
2. tel ΔC_p = mean tel.C_T (calibrator) – mean tel.C_T (sample)
3. 36b4 ΔC_p = mean 36b4.C_T (calibrator) – mean 36b4.C_T (sample)
4. the values for efficiency of each PCR is then raised to the power of the respective ΔC_p , and the ratio of telomere:36b4 calculated:
5.

$$\text{Ratio} = \frac{\text{Efficiency (tel)}^{\Delta Cp\,(tel)}}{\text{Efficiency (36b4)}^{\Delta Cp\,(36b4)}}$$

Thus the relative expression ratio is calculated only from the efficiencies and the C_T of an unknown sample versus a control. This method does not require a standard curve to be used in each and every run, but does make the assumption that while the efficiencies of reactions may differ *between* PCRs (ie between the 36b4 and telomere PCRs), the efficiency of amplification for each sample within a PCR is the same.

Comparative Quantification (CQ)

Comparative Quantification (Corbett Research, Cambridge, UK) provides a recent advance on the Pfaffl method. As discussed above, Pfaffl assumes that the amplification of each and every sample will be the same within a PCR reaction (but not necessarily 2), but this is not always true. CQ estimates the amplification efficiency of every sample within a run, and calculates an average value that is used in the subsequent analysis. In order to quantitate the method determines the "Take-Off" value for each sample (this is equivalent to the C_T value estimated in the previous methods).

To calculate the Takeoff point, the second derivative of the raw data is taken in order to identify the peak reaction velocity (Figure 2.12). This is the peak of the exponential reaction and occurs shortly after the Takeoff of the reaction. The Takeoff point cannot be determined exactly, but is estimated by finding the first point to be 80% below the peak level.

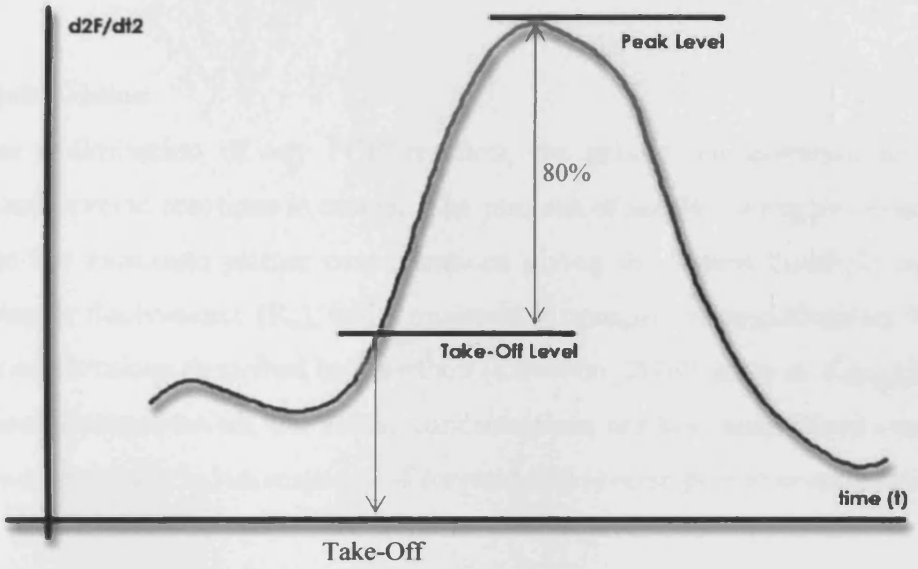


Figure 2.12: Comparative Quantification. The take-off point is determined for each sample by extrapolating back 80% from the peak reaction velocity. (Image adapted from Rotogene Version 6, Corbett Research).

Based on the Takeoff point and the reaction efficiency, the method calculates the relative concentration of each sample compared to the Calibrator (referred to hereafter as the CQ Ratio).

Example of analysis

1. The Takeoff points of each sample are calculated by examining the second derivative peaks.
2. The average increase in raw data, 4 points following the Takeoff, is calculated and used to estimate the amplification efficiency.
3. Outlier amplifications are removed to account for noise in background fluorescence.
4. The non-outlier amplifications are averaged to become a run "Average Amplification".
5. The average TakeOff point is calculated for all replicates of the Calibrator.

6. The relative concentration for a sample is calculated as:

$$\text{Amplification}^{(\text{Calibrator TakeOff} - \text{Sample TakeOff})}$$

Given the advantages of Comparative Quantification, this method of analysis was used hereafter.

Assay Optimisation

As in the optimisation of any PCR reaction, the primer concentration in both the forward and reverse reactions is crucial. The purpose of the following procedure was to determine the minimum primer concentrations giving the lowest threshold cycle (C_T) and maximum fluorescence (R_n), while minimizing nonspecific amplification. While the primer concentrations described by Cawthon (Cawthon, 2002) serve as a guideline as to the required concentrations, the actual concentrations are best determined empirically. Thus it was necessary to run matrices of forward and reverse primer concentrations.

Telomere PCR

A matrix of 4 concentrations (nM) of each primer was set up, giving a total of 16 primer combinations (Table 2.4)

Table 2.4: Telomere Primer Combinations (nM)

		Tel 2			
		180	**360**	**540**	**900**
Tel 1	**135**	135/180	135/360	135/540	135/900
	270	270/180	270/360	270/540	270/900
	540	540/180	540/360	540/540	540/900
	810	810/180	810/360	810/540	810/900

The final reaction volume was 25ul, comprising 1X Sybr Green PCR Mastermix, 25ng template, and primers at required concentration. The primer sequences (written 5'→3') were: tel 1, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT; tel 2, TCCCGACTATCC CTATCCCTATCCCTATCCCTATCCCTA. All PCRs were performed on the ABI Prism® 7900HT Sequence Detection System (Applied

Biosystems, Foster City, CA). The thermal cycling profile began with at 95°C incubation for 10 minutes to activate the AmpliTaq Gold DNA polymerase. There followed 40 cycles of 95°C for 15s, 58°C for 1 min. ABI Sequence Detection Software (SDS) version 2.1 was then used to determine C_T values for each sample run. During the optimisation stage all samples were run in triplicate. A no-template control (NTC) for each primer combination was also amplified.

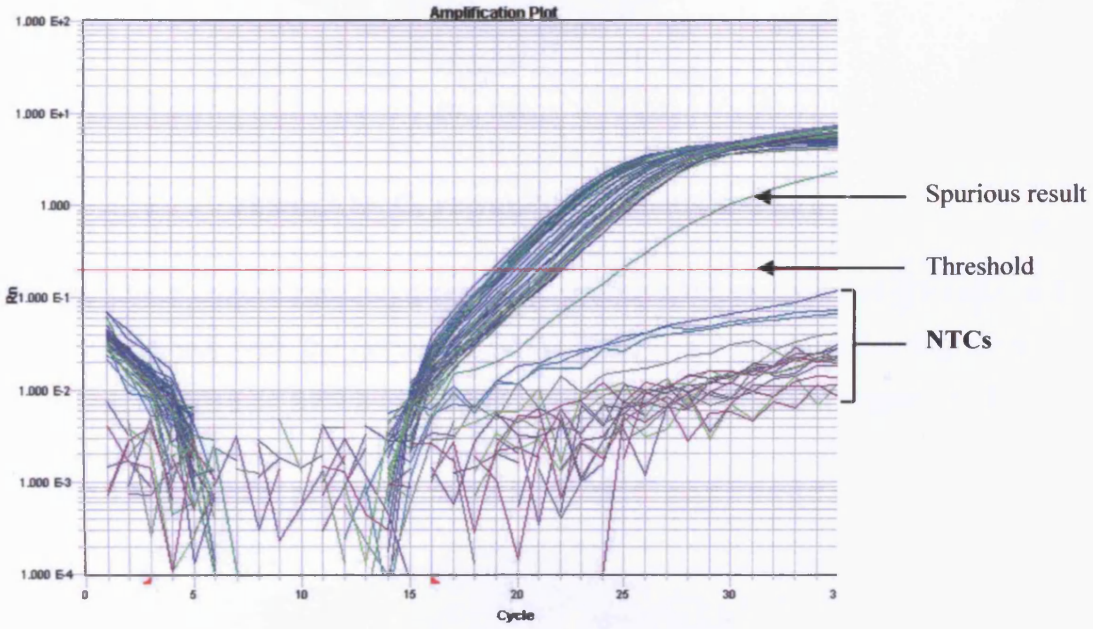


Figure 2.13: Amplification Plot. All combinations amplified with a relatively narrow range of cycles, with one spurious sample (green line). The no-template controls (NTCs) all remain below the threshold (red line).

The amplification plot in Figure 2.13 clearly demonstrates that all primer combinations tested produce good results; the exponential phase of the reaction began between cycles 15 and 17 for all samples. The exception was the green line that lags behind the other samples; this was a spurious result as the other two samples of the triplicate were approximately identical in terms of C_T and fluorescence. The NTCs did not amplify, with only the expected background noise evident. Figure 2.14 shows the average C_T value for each primer combination in the matrix

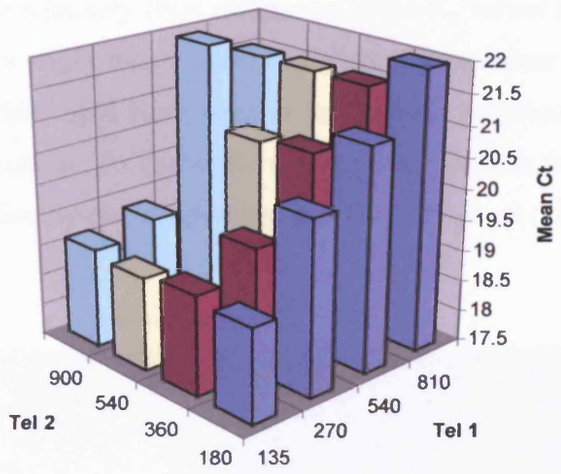


Figure 2.14: C_T versus Primer Combination.

Clearly, the lower concentrations of the Tel 1 primer produced a lower C_T value at all Tel 2 concentrations, with the exception of the 270/540nM combination. However, the C_T value does not give an indication of the R_n value for the reaction; Figure 2.15 shows the average R_n for each combination.

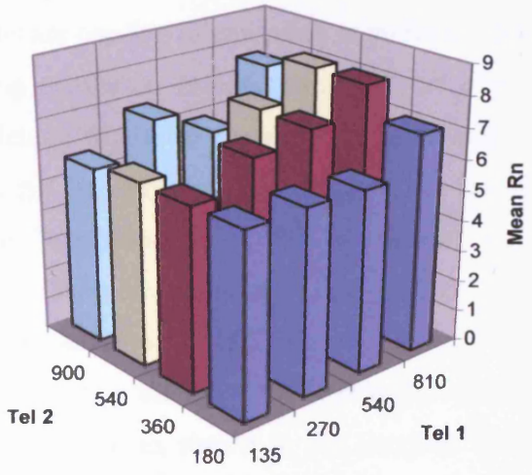


Figure 2.15: R_n versus Primer Combination.

There appeared to be relatively little difference in the R_n values across the matrix. As expected, there was a slight trend towards slightly higher values at the higher primer concentrations, but this could have been at the expense of primer-dimer and/or non-specific product formation. To check for non-specific products and primer-dimers the samples were separation by electrophoresis on a 2% agarose gel (Figure 2.16).

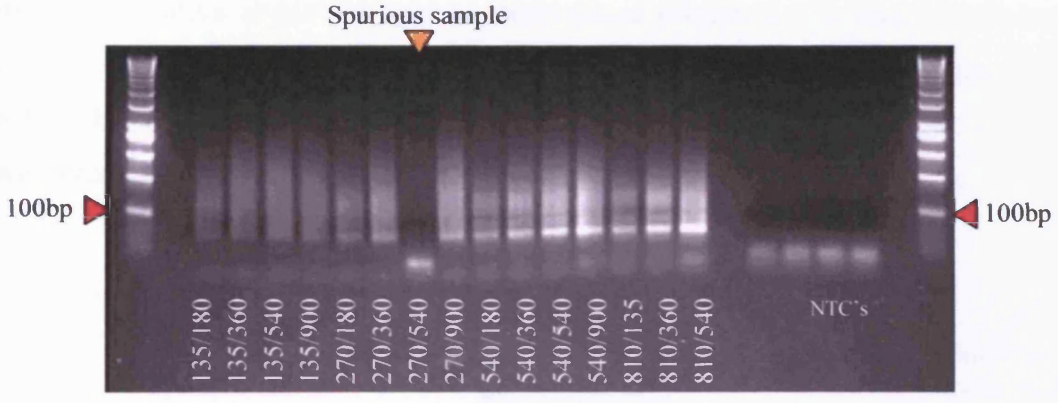


Figure 2.16: 2% Agarose Gel Electrophoresis of telomere PCR products. Each lane shows the first sample from each set of triplicates for each primer combination.

The Tel 1/Tel 2 primers are capable of annealing at multiple sites along the length of the telomere and initiating synthesis. Consequently, it was expected that a number of products of different length would be generated, and this was reflected in the smearing seen on a 2% agarose gel (see Figure 2.16). The greatest intensity was at the smallest predicted possible size, 76bp (the sum of the two primers), and progressively faded to approximately 600bp. There was evidence of minimal primer-dimer formation, particularly in the no-template controls (NTCs).

It is also important to note that the 270/540nM primer combination on the gel gave a spurious result (orange arrow), as shown in the previous Amplification Plot (Figure 2.13). It was also possible to verify the presence of a single product using the ABI 7900HT, as the machine supports dissociation curve analysis, made possible through the use of the fluorogenic SYBR Green 1 double-stranded DNA binding chemistry. Amplification products usually melt quickly and thoroughly at a temperature that is characteristic of the product; analysis of the curve with respect to temperature results in a sharp, easily distinguishable peak. To generate the data, the 7900HT performs a programmed temperature "ramp" in which it slowly elevates the temperature of the

plate over several minutes. The binding characteristic of SYBR Green 1 allows the machine to monitor the hybridisation activity of any products present in the sample; during the run the decrease in SYBR Green fluorescence resulting from the dissociation of dsDNA is recorded. The raw data is normalised and the SDS software then computes the first derivative of the normalised data for each reading taken during the ramp; this is the rate of change in fluorescence as a function of temperature. The negative of the resulting derivative is plotted against temperature (Figure 2.17), thus visualising the change in fluorescence at each temperature interval. The T_m of the target nucleic acid can then be determined from the graph by identifying the maximum rate of change, represented by the peak of the graph.

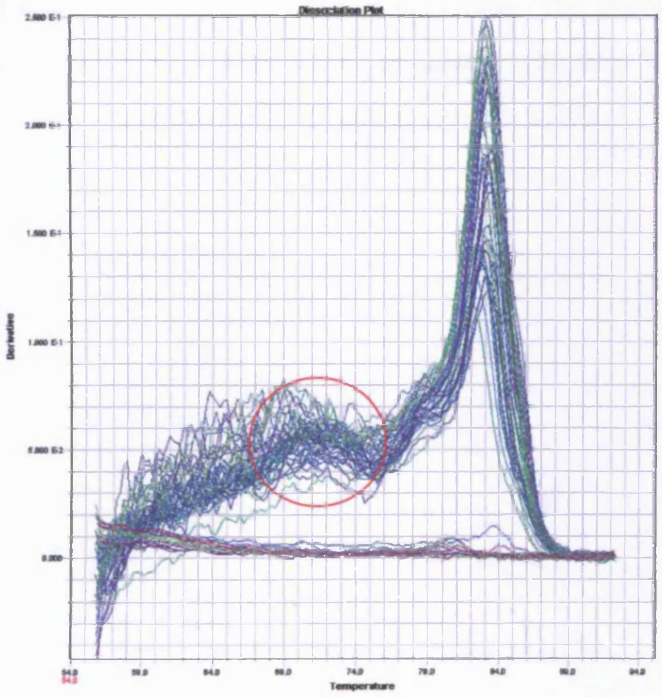


Figure 2.17:
Telomere Dissociation Plot. There is a single, sharp peak at slightly less than 84°C, reflecting the most intense band seen on the gel image, but the base of the peak is relatively broad due to products of varying length. There is also confirmation of the presence of primer-dimer, as indicated by the broad "hump" around 69°C (circled). The NTC samples demonstrate the absence of contamination.

As expected from examination of the products analysed on the agarose gel there was a single, sharp peak for each of the samples, and no evidence of contamination. Note that the degree of "noise" around the primer-dimer peak depends on the primer concentrations.

36b4 PCR

The optimisation procedure was also carried out using a matrix of forward and reverse primers for the 36b4 (data not shown). Thus, for both the telomere and 36b4 reactions I examined a number of primer concentrations in an effort to determine, empirically, the combination resulting in maximum fluorescence and minimum non-specific products at the lowest C_T possible. The optimal combinations were 270/900nM (telomere PCR) and 300/500nM (36B4 PCR), and were used hereafter in all experiments.

Assay validation

Inter-assay variation

In order to assess inter-assay variation, the assay was performed twice on identical samples, with each analysed in duplicate. The correlations in raw C_T values for the separate PCR reactions were calculated (Figures 2.18 & 2.19), in addition to the correlation between final CQ ratios (Figure 2.20).

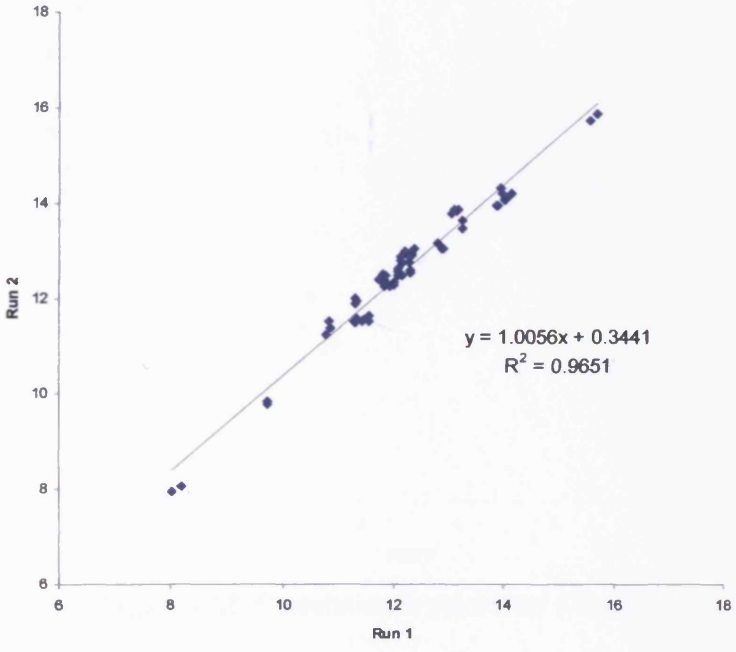


Figure 2.18. Correlation in CT values for the telomere PCR

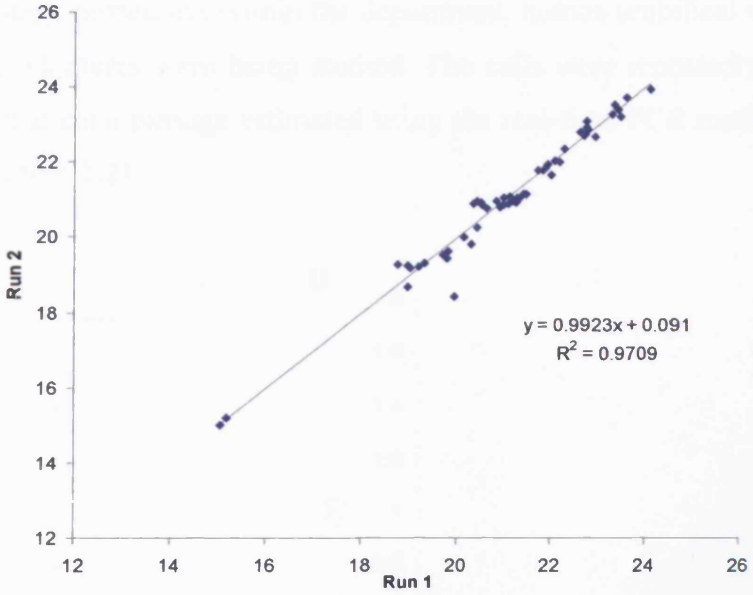


Figure 2.19. Correlation in C_T values for the 36b4 PCR

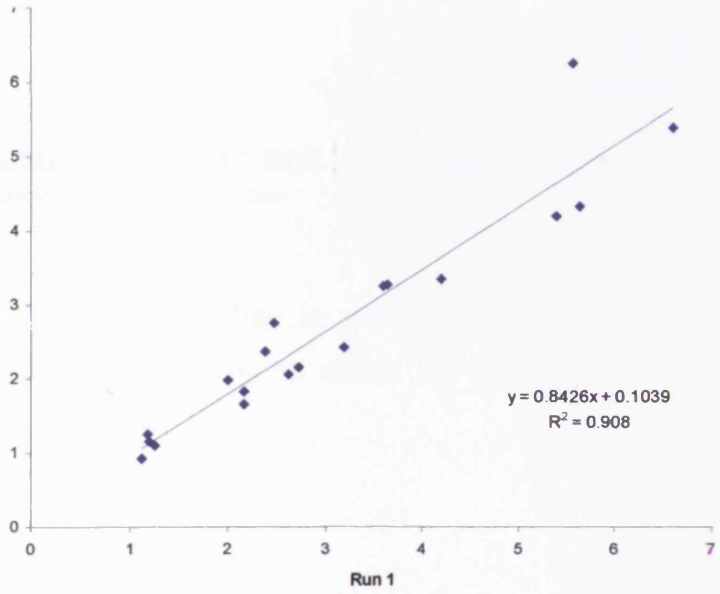


Figure 2.20. Correlation in calculated CQ ratios.

Figures 2.18 and 2.19 demonstrate the strong inter-run correlations in raw C_T values across repeat experiments. The correlation between the CQ values (Figure 2.20) is slightly lower, but this is to be expected given that the the CQ is a ratio, calculated from average C_T values in each separate PCR.

Validation using HUVECs:

In a separate study carried out within the department, human umbilical vein endothelial cell (HUVEC) telomeres were being studied. The cells were repeatedly passaged, and telomere length at each passage estimated using the real-time PCR method. The results are shown in Figure 2.21.

A.

Passage	*CQ*
5	
6	1.63
7	1.43
8	1.33
9	
10	1.48
11	1.22
12	
13	0.94
14	1.12
15	1.09
16	1.02
17	1.19

B

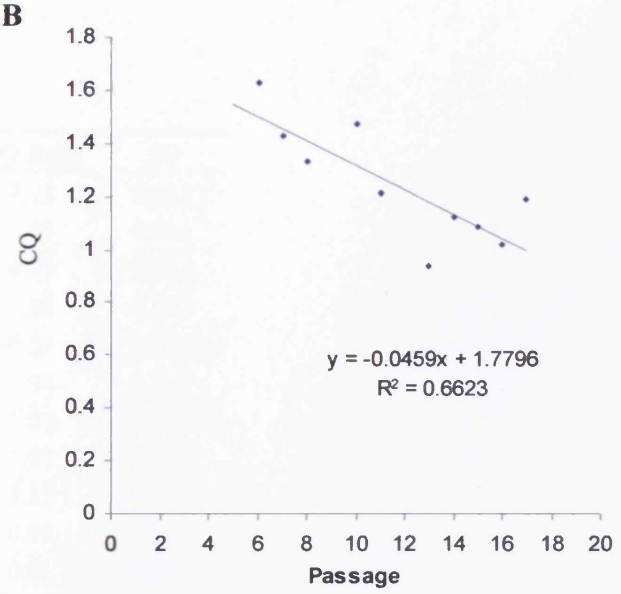


Figure 2.21: Validation using HUVECS. A. Raw data. **B.** Comparative Quantitation, plotted as a function of passage number.

The HUVECs show a clear decline in ratio with increasing passage (r = 0.81), illustrating the ability of the assay to detect the shortening of the telomere with increasing passage number.

Correlation with TRF Analysis

To further validate the real-time PCR method, it was decided to assay (in triplicate) a number of samples already analysed using the TRF length method. This allowed examination of the correlation in telomere length estimated from both methods. The samples were chosen to cover a range of mean TRF lengths (5.54 – 7.58kb) and ages (32 – 54 years). The results are summarised in Table 2.5, and Figure 2.22.

Table 2.5: Assay Validation

Sample	*Age (years)*	*mean TRF (kb)*	*CQ Ratio*	*SD*
1	50	7.02	1.21	0.025
2	39	6.58	0.85	0.004
3	49	6.91	0.95	0.081
4	48	5.54	0.58	0.002
5	53	6.45	0.81	0.014
6	49	6.09	0.94	0.117
7	48	6.75	1.01	0.087
8	52	6.46	1.07	0.102
9	50	6.78	1.17	0.023
10	47	6.53	0.92	0.031
11	54	6.74	0.91	0.024
12	47	6.83	1.18	0.093
13	32	7.58	1.28	0.080
14	52	6.49	0.62	0.103
15	51	6.61	1.11	0.063
16	48	6.17	1.21	0.094
17	38	6.89	1.15	0.005
18	43	6.92	1.14	0.063

As the intra-run variability for the triplicates was low (as indicated from the SD values), subsequent experiments were carried out with samples in duplicate. This allowed a greater number of samples to be analysed per run. If values for a particular sample varied (i.e. by more than 0.2 of a cycle), the sample was re-analysed in triplicate.

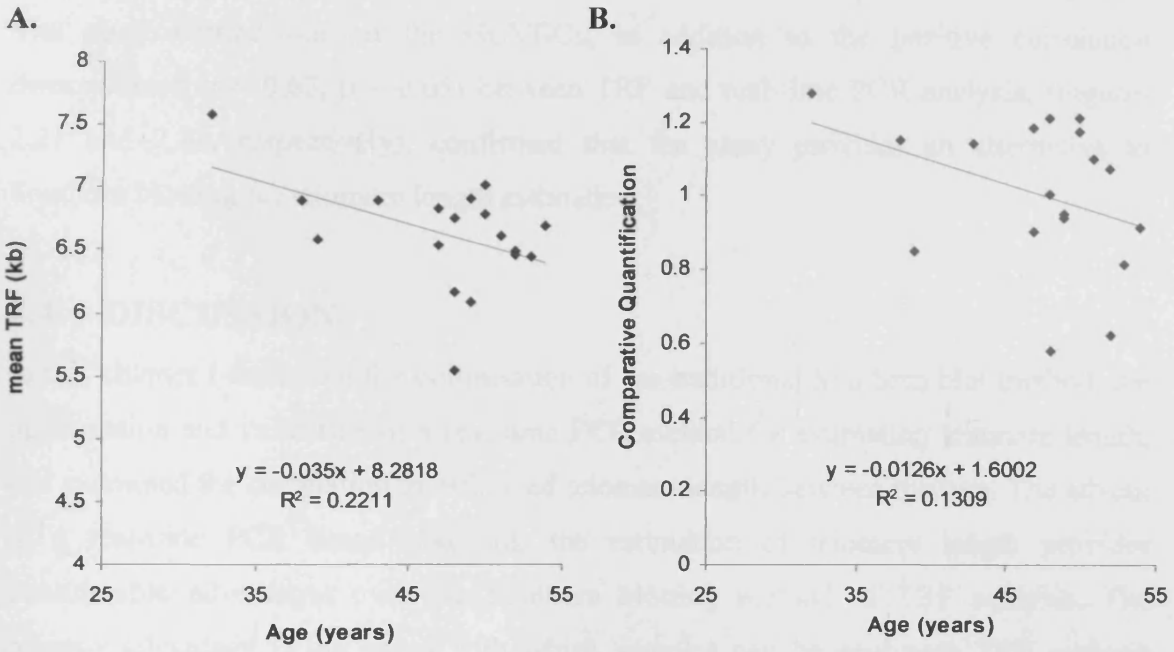


Figure 2.22: Assay Validation. A. Telomere length estimated using TRF analysis. **B.** Telomere length estimated using Real-time PCR analysis (CQ).

Both methods of analysis have demonstrated the expected age-related decline in telomere length. Figure 2.23 illustrates the correlation between the two methods.

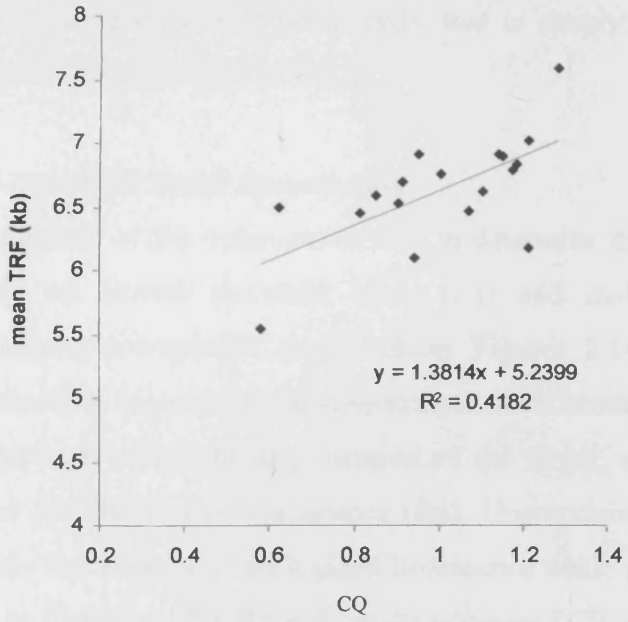


Figure 2.23: Correlation between TRF and Real-Time PCR Analysis. There is a clear, positive correlation (r=0.65, p=0.05) between telomere length, as determined using the two different methods.

The assay carried out on the HUVECs, in addition to the positive correlation demonstrated ($r = 0.65$, $p = 0.05$) between TRF and real-time PCR analysis, (Figures 2.21 and 2.23, respectively), confirmed that the assay provides an alternative to Southern blotting for telomere length estimation.

2.4 DISCUSSION

In this chapter I described the optimisation of the traditional Southern blot method, the optimisation and validation of a real-time PCR method for estimating telomere length, and examined the correlation in estimated telomere length between the two. The advent of a real-time PCR based assay for the estimation of telomere length provides considerable advantages over the Southern blotting method of TRF analysis. The primary advantage is the speed with which samples can be analysed; TRF analysis consists of restriction enzyme digest (overnight), quantification, agarose gel electrophoresis (overnight), Southern blotting (overnight), pre-hybridisation, hybridisation, exposure to autoradiographic film (12-72hrs), exposure to phosphorimager plate (12-72hrs), and finally the TRF analysis itself. By contrast, real-time PCR analysis yields a result for a comparable number of samples in approximately 4 hours. In addition, the requirement for cnly 10-20ng of DNA, instead of the 2-5μg required for Southern blotting, means telomere length can be analysed in cells types, such as circulating endothelial cells, that is simply impossible with southern blotting due to limiting amounts of DNA.

Real-time PCR Assay Optimisation

The purpose of the optimisation was to determine the minimum primer concentrations giving the lowest threshold cycle (C_T) and maximum fluorescence (Rn), while minimizing non-specific amplification. Figures 2.14 and 2.15 (C_T, and R_n v primer combination, respectively) demonstrate how different combinations of primers can have a significant effect on amplification of the target, as indicated by the threshold (C_T) values and the mean fluorescence (Rn). Unsurprisingly, the primer combinations that provide the lowest C_T and highest florescence while minimising primer-dimer are those used by Cawthon: 270/900nM for the telomere PCR, and 300/500nM for the 36b4 PCR. The method of analysis for relative quantification is an important choice that requires careful consideration. ABI recommend the comparative ($\Delta\Delta C_T$) method, which assumes

ideal amplification efficiency with a doubling of product every cycle, thus the fold change can be calculated from the formula $2^{-\Delta\Delta Ct}$ (Livak, 1997; Livak and Schmittgen, 2001). However, it has become very apparent that assuming ideal amplification can be a mistake leading to inaccurate quantification (Liu and Saint, 2002), resulting in refinement to the analysis method. Pfaffl has outlined a modification that takes into account the relative efficiencies of the two reactions, as determined from a set of standard curves, resulting in more accurate results (Pfaffl, 2001). This method does not require a standard curve to be used in each and every run, but does make the assumption that while the efficiencies of reactions may differ *between* PCRs (ie between the 36b4 and telomere PCRs), the efficiency of amplification for each sample within a PCR *is* the same. Again, this is not necessarily the case, as samples of DNA may possess secondary structure or contain PCR inhibitors carried over from the DNA extraction. Corbett Research attempt to address this issue by estimating the efficiency for every sample within a run, rather than using values taken from a set of standard curves. Thus the relative expression ratio (CQ Ratio) is calculated only from the efficiencies and the "Take-Off" point of an unknown sample versus a control.

Real-time PCR Assay validation

In order to validate the PCR-based method of telomere analysis three simple experiments were carried out. 1) A slection of samples covering a wide range of TRFs were chosen, and analysed in two separate experiments to assess inter-assay variations. Strong inter-run correlations were demonstrated for the 36b4 PCR, the telomere PCR, and the calculated CQ ratios. This experiment demonstrated that the assay is highly reproducible. 2) HUVECs are a primary cell line that are known to experience telomere attrition (Hastings *et al.*, 2004), thus serially passaged cells have progressively shorter telomeres. Assaying cells from passages 6 -17 demonstrated a strong, negative correlation between CQ ratio and passage number. 3) A number of samples, previously analysed using the TRF method, were selected to cover a range of mean TRFs (5.54 – 7.58kb), and ages (32 – 54 years). All samples were assayed, in triplicate, using the PCR method, and the correlation between the two methods determined. While I demonstrated a strong correlation ($r = 0.65$), this value is not as strong as that demonstrated by Cawthon ($r = 0.82$). The most reasonable explanation for this is that Cawthon analysed a much wider range of ages (5 - 94 years) and mean TRF values

(approx 5 – 10.5kb). By using a comparable range of ages I may have been able to demonstrate a stronger correlation.

In addition, human telomeres consist of multiple TTAGGG repeats, and a region of sub-telomeric repeats characterised by varying numbers of the hexameric sequence. TRF analysis utilises an 18-mer probe that can hybridise to both the "true" telomeric repeats, and the sub-telomeric repeats, a region that can show considerable variation. In contrast, the PCR-based method only measures the "true" telomere, and so differences in estimated length between the two methods may simply be a reflection of the varying sub-telomeric region in the cohort analysed. If the sub-telomeric region shows a wide variation in length, and TRF analysis measures this region, it is possible that two samples giving the same estimated mean TRF value may, in fact, be quite different. This highlights an additional potential advantage of using the real-time PCR based method of telomere analysis.

ASSOCIATION OF TELOMERE LENGTH WITH PREMATURE MYOCARDIAL INFARCTION

3.1 INTRODUCTION

As discussed in Chapter 1, Samani *et al.* (Samani *et al.*, 2001) had demonstrated shorter telomeres in the white blood cells of patients with severe triple vessel disease, compared to age and sex-matched controls. While this was a small study and did not examine the effect of other cardiovascular risk factors on telomere length, these results raised the interesting possibility that CHD may be a result of premature biological ageing. This, along with the fact that 1) a positive family history is a well-recognised risk factor for CHD, and that 2) telomeres have a significant genetic determinant, lead to the additional hypothesis that the inheritance of shorter telomeres may also explain, in part, the increase in familial risk in CHD.

Atherosclerosis is the most common cause of CHD. CHD and myocardial infarction (MI) are closely associated with the risk factors of atheroma: dietary factors, obesity, familial predisposition, hypertension, physical activity, environmental factors, stress and smoking (Olsen, 1991). Severe triple vessel disease, as examined by Samani *et al.* (Samani *et al.*, 2001), represents atherosclerosis in a very advanced form; if shorter telomeres are inherited, and do indeed lead to premature biological ageing and increased risk of CHD, then we would expect to see differences in length at an earlier age in those with CHD. An important clinical consequence could be an increased risk of premature myocardial infarction. Therefore, in this study I examined whether there is an association between telomere length and premature myocardial infarction in a large cohort of patients and age and sex-matched controls, and also investigated whether any association found could be explained by other, more well-established risk factors for CHD.

3.2 METHODS

3.2.1 Subjects

3.2.1.1 Cases

The cases comprised 203 subjects recruited retrospectively from the registries of three coronary care units based in Leicester (Leicester Royal Infirmary, Leicester General Hospital and Glenfield General Hospital). All had suffered a myocardial infarction according to World Health Organisation criteria before the age of 50. Diagnosis was verified by inspection of hospital records. At the time of participation all case subjects were at least 3 months from their acute event and in a clinically stable condition. Subjects were excluded if there was evidence of thrombophilic disorders such as Protein C and S Deficiency.

3.2.1.2 Controls

The control cohort comprised 180 subjects with no personal or family history of premature coronary heart disease, matched for age, sex and current smoking status with the cases. Control subjects were recruited from three primary care practices located within the same geographical area

To avoid the problem of genetic heterogeneity only Caucasian patients who were born within Northern Europe and whose parents were also born within Northern Europe were studied. The study was approved by the Leicestershire Health Authority Ethics Committee (Ethics Reg. No. 5506) and all subjects provided written informed consent.
The initial purpose of the study was to determine if patients suffering a premature MI demonstrate consistent abnormalities of platelet reactivity, and if the abnormalities are due to inheritable variation in platelet receptor glycoprotein genes.
All subjects were recruited by Dr. RK Singh.

3.2.2 Measurements

A standard questionnaire was filled in on all subjects regarding personal and family history and drug therapy. Subjects were categorised as suffering from hypertension and diabetes on the basis of reported history. Smoking status was defined as current smoker, ex-smoker and non-smoker. Serum cholesterol was measured using standard reagents (7D62-01) on the AEROSETTM system (Abbott Laboratories, IL, USA). White cell

count was measured in EDTA blood using a Beckman Coulter® AC.T diffTM counter. C-reactive protein was measured using the ultra-sensitive Olympus CRP Immunoassay on an Olympus AU 100 Analyser. Fibrinogen was measured by the Clauss method on a Sysmex CA-1000 analyser. Homocysteine was measured by HPLC with fluorescence detection using a commercially available kit (Chromesystems Limited, UK).

3.2.3 Terminal restriction fragment (TRF) analysis

TRF analysis was performed as detailed in Chapter 2, and blinded to the clinical data.

3.2.4 Statistical analysis

Characteristics of cases and controls were compared using unpaired t-test for continuous variables and chi-squared test for categorical variables. The effects of age, gender, case/control status and other individual risk factors on mean TRF length were assessed using regression models, with adjustments as described in the text. Mean TRF length as a risk factor for MI was assessed using logistic regression. All analysis was carried out in Stata Statistical Software (Release 7.0; Stata Corporation, College Station TX; 2001). Advice and assistance was provided by Professor JR Thompson.

3.3 RESULTS

3.3.1 Demographics

The demographic characteristics of the subjects are shown in Table 3.1.

Table 3.1: Demographics of cases and controls

Demographic	*Cases (n = 203)*	*Controls (n = 180)*	*p-value*
Age (years)*	46.8 (6.2)	47.2 (5.9)	0.483
M:F (%)*	85:15	86:14	0.934
Age at time of MI	42.3 (5.7)	N/A	-
Current/Ex/non–smokers (%)*	21:61:18	17:33:50	<0.0001
Hypertension (%)	28.1	8.3	<0.0001
Diabetes (%)	10.8	1.1	<0.0001
Positive family history#	59.6	N/A	-
White cell count (x 10^6/ml)	6.85 (0.15)	5.43 (0.10)	<0.0001
Plasma cholesterol (mmol/l)	4.92 (1.28)	5.22 (1.02)	0.015
Plasma fibrinogen (mg/dl)	3.14 (0.74)	2.80 (0.57)	<0.0001
Plasma C-reactive protein (mmol/l)∞	2.55 (5.68)	1.92 (1.99)	0.787
Plasma homocysteine (mmol/l)	12.25 (0.29)	11.88 (0.31)	0.377

Data are shown as percentages for categorical variables and means (standard deviations) for continuous variables. N/A – not applicable. * characteristics used for matching (note that for smoking, this was for current smoking and the significant p value reflects the difference in the number of previous and non-smokers between the groups); # History of CHD in first degree relative below 65 years. ∞ The CRP distributions were skewed and the p value was calculated on log-transformed data.

Cases and controls were well matched for both age and sex. The mean age at event of the cases was 42.3±5.7 years. As expected, the presence of cardiovascular risk factors, such as diabetes and hypertension, was greater in cases than controls, and more of the cases were current or ex-smokers. Plasma cholesterol level at the time of study was significantly lower in cases and controls, but this likely reflects the fact that a high proportion of the cases (76.8 %) were taking lipid-lowering medication. White cell count and plasma fibrinogen level were significantly higher in cases compared with controls but there was no significant difference in plasma C-reactive protein or homocysteine levels. Almost 60% of cases reported a history of CHD before the age of 65 years in a first-degree relative.

3.3.2 Distribution of mean TRF lengths

The mean white cell TRF length as a function of age is shown in Figure 3.1 for both cases and controls. This figure clearly illustrates the wide degree of inter-individual variation in telomere length at any given chronological age.

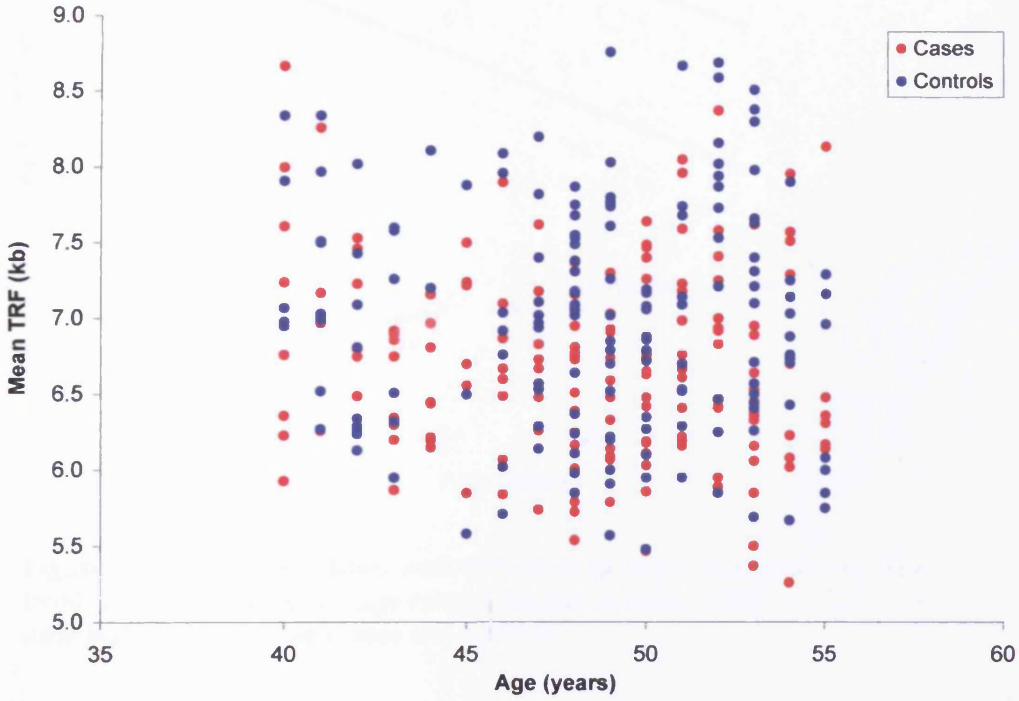


Figure 3.1: Mean TRF's of cases and controls. There is considerably inter-individual variation at all ages.

From these data, regression lines along with 95% confidence intervals were determined for both cases and controls, and are plotted in Figure 3.2. ....

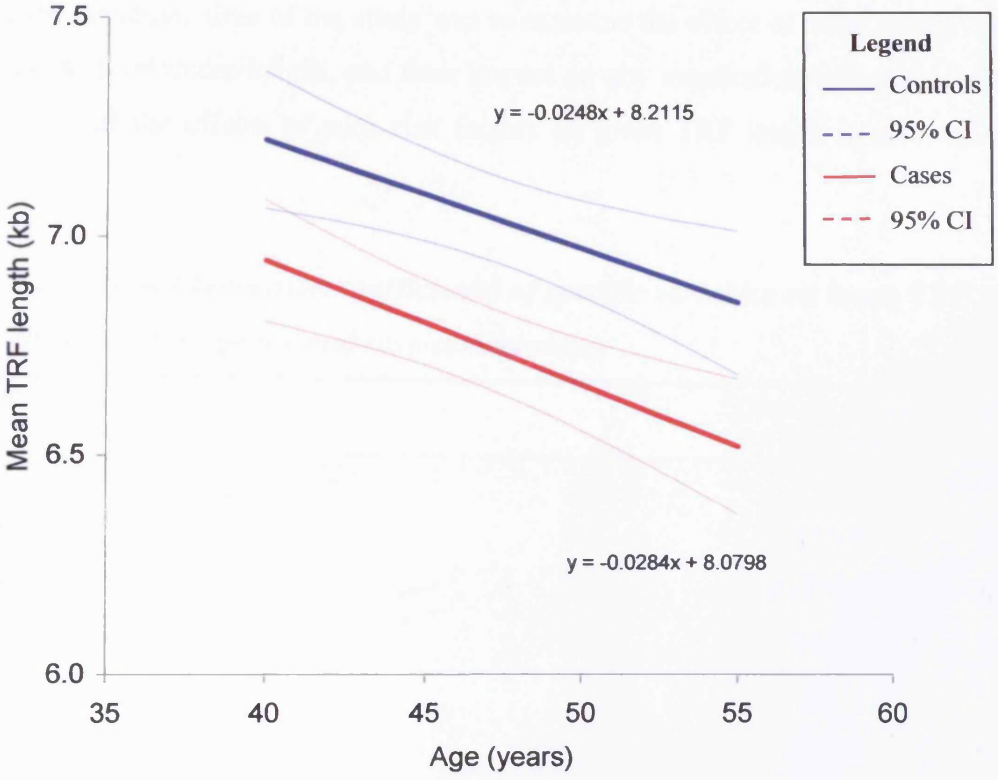


Figure 3.2: Regression lines and 95% CIs in cases and controls. The trend lines demonstrate an age-related decline in mean TRF length, and a clear partitioning between cases and controls.

Despite the relatively narrow age range, highly significant ($p < 0.0001$) decreases in mean white cell TRF length with increasing age were observed in both groups. The decrease per year was 28.3±7.0 base pairs (bp) in cases and 24.8±9.3 bp in controls. This difference was not significant ($p = 0.757$). Age adjusted mean TRF lengths for cases and controls split by gender were 6.77±0.17 kb for male cases, 6.62 ± 0.18 kb for female cases, 7.05±0.16 kb for control males and 7.02± 0.11 kb for control females. There was no independent effect of gender on mean TRF length ($p = 0.327$). However, taking age and gender into account, the mean TRF length of cases was significantly lower compared to controls (difference 299.7±69.3 bp, $p < 0.0001$).

3.3.3 Impact of other risk factors on mean TRF length

One of the principal aims of the study was to examine the effect of other cardiovascular risk factors on telomere length, and their impact on any association with premature MI. An analysis of the effects of such risk factors on mean TRF length is summarised in Table 3.2.

Table 3.2: Effects (Regression coefficients) of specific variables on mean TRF length after adjustment for age/sex and case/control status

Variable	*Effect on mean TRF length (bp)*	*p*
History of hypertension (n = 72)	-99.2 (92.7)	0.285
History of diabetes (n = 24)	+1.3 (146.8)	0.993
Smoking: Current (n = 72)	-63.8 (103.8)	0.539
ExSmoker (n=185)	+8.2 (84.6)	0.923
Per pack year	-2.3 (2.0)	0.264
Per 1 x 10^6 higher white cell count	-17.0 (20.3)	0.404
Per mmol/l higher fibrinogen	-21.1 (54.5)	0.699
Per mmol/l higher CRP	-4.4 (8.0)	0.583
Per mmol/l higher homocysteine	+12.2 (8.5)	0.149
Positive family history (CHD in 1° relative < 65 years)*	-114.8 (88.4)	0.195
Positive family history (MI in 1° relative < 50 years)*	-152.5 (94.3)	0.107

Standard error shown in brackets. Negative value indicates that the variable was associated with shorter mean TRF length. * Only in cases, see legend to Table 3.1 (Demographics) for definition.

A history of hypertension was associated with mean white cell TRF length that was almost a 100 bp shorter but this did not reach statistical significance. No independent effects were found for either a history of diabetes or smoking. There was also no significant relationship between mean TRF length and either white cell count or plasma levels of C-reactive protein, fibrinogen or homocysteine. Interestingly, in case subjects, there was a trend towards shorter mean TRF length in those with a positive history of premature CHD (especially in those with a first degree relative with an MI before age of 50) although this did not reach significance (Table 3.2). Input of all of these factors into a logistic regression analysis did not affect the relationship between case status and shorter TRF length (difference 315.7±84.9 bp, p = 0.0002).

3.3.4 Telomere length and risk of MI

Figure 3.3 displays the data described previously in a different format: the range of TRF's are divided into quartiles (Q1 being the longest, and Q4 the shortest), and the odds ratio for risk of MI for each quartile are plotted.

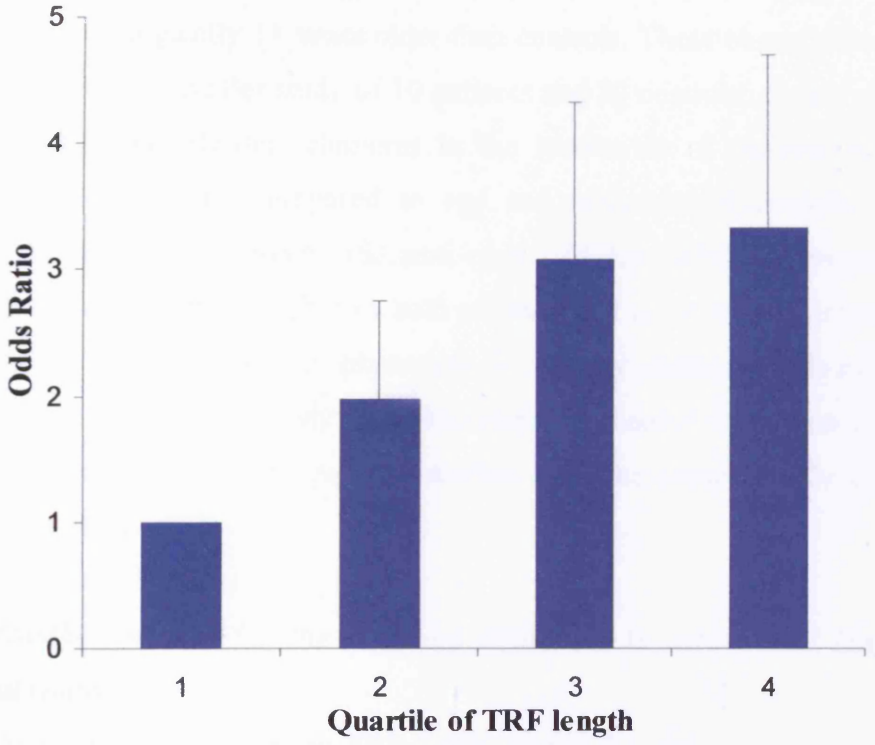


Figure 3.3: Odds ratio of risk of MI. in different quartiles of mean TRF length compared with the highest quartile (1).

Compared with subjects in the highest quartile for TRF (set at 1), the odds ratios were 1.63 (95% CI, 0.91-2.92, p = 0.102), 3.27 (95%CI, 1.79-5.97, p < 0.0001) and 3.34 (95%CI, 1.50-7.43, p = 0.002), respectively in subjects in the second, third and lowest quartile of mean TRF length. These data clearly display a graded relationship between telomere length and risk of MI.

3.4 DISCUSSION

In this study I have shown that the telomeres of white blood cells obtained from the peripheral blood of patients who have suffered from a premature MI are significantly shorter than those from age and sex-matched controls. The average decline in TRF

length per year was 26.4bp, a value that is within the range reported previously in the literature (Samani *et al.*, 2001; Wynn *et al.*, 1998). Given this decline, and the observed difference in mean TRF length between cases and controls (299.7 bp), the biological age gap between cases and controls is over 11 years, despite the relatively narrow chronological age range of the subjects. To put this into simpler terms, the results imply that cases are biologically 11 years older than controls. These observations support the results reported in a smaller study of 10 patients and 20 controls, carried out within our group, which found shorter telomeres in the leucocytes of patients with advanced coronary atherosclerosis, compared to age and sex-matched controls with normal coronary arteries at angiography (Samani *et al.*, 2001a). While the present study has examined a much greater number of both subjects and controls, it is important to note that the precise cardiovascular phenotype is slightly different: advanced coronary atherosclerosis in the first study and premature myocardial infarction in the present study. Of course, the two lie on a continuum with the present study examining the disease at an earlier stage.

3.4.1 Possible reasons for the observed difference in mean TRF length between cases and controls

There was a wide range of mean TRF values across the entire age range (Figure 3.1). Indeed the average range at any particular age was approximately 3kb. The regression lines and 95% confidence intervals plotted in Figure 3.2 clearly show a partitioning, and an age-related decline in telomere length of 26bp/year. There was no independent effect of gender on telomere length, an observation that does not concur with other studies that have shown females to have shorter telomeres than men (Benetos *et al.*, 2001; Cawthon *et al.*, 2003; Jeanclos *et al.*, 2000). The lack of gender effect in the present study may be a result of the small number of women in the study.

There are three possible explanations for the observed difference in mean TRF length between cases and controls.

The first is that the observation is an artefact, but the strength of the association demonstrated between shorter mean telomere length and risk of MI argues against this possibility.

The second potential explanation is that the subjects all inherited telomeres of a similar length, but differing rates of attrition between cases and controls have resulted in the

300bp difference observed. Therefore, this study examined the effect of both recognised risk factors for CHD, and factors known to influence the rate of telomere attrition, on telomere length. A history of hypertension was associated with slightly shorter telomeres, although this did not reach statistical significance, and no effect of diabetes or smoking was observed. These three factors are all independent risk factors for CHD, and are potential sources of insult to the vascular endothelium that could result in increased turnover and the subsequent attrition of the telomere. There is also compelling data implicating low-grade inflammation (Ross, 1999) and increased oxidative stress (Harrison, 1998) in the pathogenesis of coronary atherosclerosis. White cell counts (Hansen *et al.*, 1990) as well as plasma levels of inflammatory markers such as CRP (Zhou and Elledge, 2000) and fibrinogen (De Backer *et al.*, 2002; Heinrich *et al.*, 1994) have been associated with CHD and have been shown to be strong predictors of future events (Packard *et al.*, 2000; Ridker *et al.*, 1997). It has also been demonstrated that, *in vitro*, telomere attrition is accelerated by oxidative stress (von Zglinicki, 2002; von Zglinicki, 2000) and homocysteine (Xu *et al.*, 2000), itself a recognised risk factor for atherosclerosis (Eikelboom *et al.*, 1999). The shorter telomeres observed in cases may simply reflect an increase in replicative stress placed on the white blood cells as a result of the chronic inflammation that likely precedes atherosclerosis. To explore this possibility I assessed the impact of several relevant parameters, and demonstrated that both fibrinogen level and white cell count were significantly higher in cases than controls, and there was a non-significant trend toward higher CRP levels. However, neither these factors, nor the plasma homocysteine level had a significant effect on mean TRF length. Logistic regression analysis found that none of these factors affected the association of shorter mean TRF length with risk of MI. Indeed, adjustment for these variables and the demographic risk factors slightly increased the difference in mean TRF length between cases and controls.

The third, and most interesting possibility is that cases and controls have *inherited* telomeres of different lengths, suggesting that short telomeres are causally related to CHD, rather than simply serving as a marker of disease. The fact that the rates of attrition are very similar for both groups (28.3±7.0 bp) in cases and 24.8±9.3 bp in controls), resulting in a mean difference of approximately 300bp across the age-range of the study, supports this explanation: if the regression lines were extended to the y-axis (mean TRF length), there would clearly be a difference between the two groups.

Genetic regulation of telomere length has been suggested by both twin (Slagboom *et al.*, 1994) and family studies (Jeanclos *et al.*, 2000) that have estimated heritability of telomere length of white blood cells to be 75-80%. The study carried out by Slagboom *et al.* (Slagboom *et al.*, 1994) is particularly interesting as it examined the correlation in telomere length between siblings, mono and dizygotic twins. The weakest correlation was observed in sibs, and the strongest in the dizygotic twins.

If there is a primary genetic basis for the difference then there are a number of potentially important implications. The first is that those individuals born with relatively short telomeres are at an increased risk of premature CHD, and a tendency to having shorter telomeres would also partly explain the increased familial basis of CHD. In this respect it is interesting to note that case subjects with a positive family showed a trend towards even shorter telomeres (see Table 3.2), although this did not reach significance.

3.4.2 Possible mechanisms linking telomere length with risk of MI

There is both *in vivo* and *in vitro* evidence suggesting that cellular senescence plays a role in the pathogenesis of atherosclerosis. Morphological changes consistent with senescence have been demonstrated in the endothelium overlying atherosclerotic plaques (Burrig, 1991; Davies *et al.*, 1988; Minamino T *et al.*, 2002), and these cells have been shown to express increased levels of plasminogen activator inhibitor type 1 (PAI-1) (Comi *et al.*, 1995; Xu D *et al.*, 2000). Minamino *et al.* have also demonstrated increased intracellular adhesion molecule-1 (ICAM-1) and decreased levels of nitric oxide (Minamino *et al.*, 2002) in senescent cells. These are all changes that are implicated in atherogenesis (Lusis, 2000). PAI-1 is particularly relevant to MI, as over-expression by vascular smooth muscle cells reduces their ability to migrate into the extracellular matrix, resulting in thin fibrous plaques that are prone to rupture (Schneider *et al.*, 2004).

Minamino *et al* (Minamino *et al.*, 2002) have recently shown that some of these functional changes are directly related to telomere biology in human vascular endothelial cells. When telomere dysfunction was induced by inhibition of TRF2 using a mutant lacking the TRF2-binding domain, phenotypic changes characteristic of senescence occurred and the cells exhibited increased ICAM-1 expression and decreased endothelial nitric oxide synthase (eNOS) activity (Davies *et al.*, 1988). Conversely, introduction of catalytic sub-unit of telomerase significantly extended the

life span and inhibited the functional alterations associated with senescence (Minamino *et al.*, 2002). The effect inhibition of TRF2 has on endothelial cells is not surprising, given the critical role the protein plays in maintaining the telomeric structure. By using a mutant lacking the DNA-.binding domain the protein is unable to bind the telomeric sequence; the effect of loss of TRF2 results in cell death via apoptosis or senescence, depending on the cellular background. These studies provide a plausible mechanism linking shorter telomeres to the development of atherosclerosis. This would also be consistent with the graded relationship we have demonstrated in this study: if vascular endothelial dysfunction is key in the development of atheroma, and shortening telomeres induce dysfunction, it follows that those individuals with the shortest telomeres have the greatest chance of developing endothelial dysfunction, the subsequent athermoa, and, ultimately, a clinical event.

3.4.3 Limitations of the study

There are a number of limitations that require consideration. This was a case-control study;, thus there is the potential for introducing bias: the cases in this particular study represent survivors of a MI, but up to 30% of subjects who suffer an MI do not survive the acute event. Consequently I have no telomere length data for such subjects, thus am unable to exclude the (unlikely) possibility that possessing longer telomeres adversely affects the immediate prognosis after MI, and that this explains the observed association. However, the data presented in this chapter are supported by a separate study, carried out by Cawthon *et al.* (Cawthon *et al.*, 2003); his group assessed the association between telomere length and mortality in 143 normal unrelated individuals over the age of 60 and found that individuals with shorter telomeres in WBCs at baseline had a 3.18-fold higher mortality rate from heart disease. This mortality rate for heart disease is in close agreement with that demonstrated between quartiles 1 and 4 in this study (3.34). These results support the hypothesis that telomere shortening in humans contributes to mortality in many age-related diseases, and to CHD in particular. To address the issue of potential bias it would be necessary to replicate the present data in a larger, prospective study.

The design of this study also meant I was unable to demonstrate a correlation between telomere length in leucocytes and telomere length in other tissues. If the primary hypothesis is correct, and telomere length is largely inherited, then individuals with

shorter leucocytes should also possess shorter telomeres in all other cell types. Studying telomere length in other cells presents an interesting challenge, as TRF analysis requires a large quantity of DNA, typically 4-6μg. While it is possible to obtain sufficient DNA from circulating WBCs, it is far more difficult to obtain this amount from other tissues, for example circulating endothelial cells. In order to demonstrate a correlation between WBCs and other tissues it would be interesting, particularly in the context of cardiovascular function, to determine the telomere length in healthy and diseased arterial and venous tissue, in addition to WBCs. To extend this study further, I would also like to study both healthy subjects and subjects with CHD, as, if the hypothesis is correct, apparently "healthy" cardiovascular tissue in a patient with CHD would already posses relatively short telomeres.

Mean TRF length has been estimated in the *whole* white blood cell population, and it would be interesting to know if there are differences in the telomere lengths of particular subsets of white cells. Also, TRF analysis provides only a crude measure of telomere length; there are considerable intercellular and inter-chromosomal differences (Lansdorp *et al.*, 1996) that result in the characteristic telomeric "smear". Examining the telomere lengths of individual telomeres requires alternative methods, such as PCR (Baird *et al.*, 2003) and fluorescence *in situ* hybridisation (FISH) (Batliwalla *et al.*, 2000; Ferlicot S *et al.*, 2003). These might allow us to determine if, for example, premature MI associates with the shortening of one particular telomere rather than another, as it has been proposed that the critical shortening of just one telomere is sufficient to induce senescence (Martens *et al.*, 2000).

In summary, this study has clearly demonstrated a powerful association between shorter mean TRF length in leucocytes and increased risk of premature MI that is independent of other risk factors and markers of inflammation. A graded relationship between progressively shorter telomeres and increasing risk of MI has also been observed. These observations have raised a number of important questions that require further study: 1) If the cases in this study are at increased risk due to the inheritance of shorter telomeres, then is follows that their offspring may be also have inherited shorter telomeres, and thus be at increased risk. By comparing the offspring of subjects with CHD with offspring of controls, it may be possible to demonstrate a difference in telomere length that suggests that telomere length is an inherited trait, and may explain, at least in part,

the increased familial risk. 2) This was a case-control study, and as such has certain limitations: the potential for bias, and the fact that I have been unable to examine telomere length in subjects who have died from an acute MI. To address these issues it is necessary to conduct a larger, prospective study. 3) If telomere length is an inherited trait, what is the mechanism of inheritance? By conducting a linkage analysis study of a large cohort it may be possible to identify candidate genes that influence the inheritance of telomere length. Work carried out to examine these questions is described in subsequent chapters.

TELOMERE LENGTH IN THE OFFSPRING OF SUBJECTS WITH A HISTORY OF PREMATURE MI

4.1 INTRODUCTION

In Chapter 3 I showed that the mean TRF length of subjects with premature MI was significantly shorter than that of age and sex-matched controls by approximately 300bp. A rate of loss of approximately 26bp/year implied that cases are "biologically" 11 years older than controls. By examining the effect of various factors and their effect on telomere length, the study also attempted to exclude confounding risk factors that may explain the association of mean telomere length with risk of developing CHD. The findings raised the possibility that inherited variations in telomere length may be responsible for the association. If this hypothesis is true, then studying the offspring of subjects with premature CHD may provide supporting evidence. A positive family history is an important independent risk factor for CHD (Colditz *et al.*, 1991), and the incidence of MI has been shown to be *higher* in individuals with a positive family history (Ciruzzi *et al.*, 1997). Furthermore, in male twin studies the relative risk of death secondary to CHD if the twin died of CHD before the age of 50 was 8.1 (monozygous) and 3.8 (dizygous) (Marenberg *et al.*, 1994).

The argument would be that if the offspring of subjects with CHD already possess shorter telomeres than age-matched controls, they might be susceptible to the premature development of atherosclerosis, CHD and MI. Thus, in this study I examined the healthy offspring of those individuals both with and without CHD to see if there was a partitioning in telomere lengths between the two groups at a young age, before any clinical signs of cardiovascular disease are manifest.

4.2 METHODS

4.2.1 Subjects

Offspring of Cases

Offspring of subjects with premature MI, hereafter referred to as "cases", comprised 24 subjects with a two-generational history of premature MI: parents must have suffered an MI before the age of 50, and a grandparent must have suffered an MI before the age of 60. Subjects were free from any major health problems and were not taking any

medication. All "case" offspring were children of subjects enrolled in the *Telomere Length & Premature MI Study* (see Chapter 2).

Controls

Controls comprised 24 subjects recruited from either the *Telomere Length & Premature MI Study* (n=4), or from Glenfield Hospital (n=20). Subjects had no family history of heart disease in their immediate family (parents and grandparents), were free from any major health problems, and were not taking any medications.

All subjects were young, adult male Caucasians. Subjects were recruited by Nashat Qamar and Aqib Bhatti.

4.2.2 Measurements

An accurate risk profile assessment of each subject, including smoking status, history of diabetes, hypertension and hypercholesterolaemia, was compiled using a questionnaire. A family history of MI was confirmed using a genetic pedigree chart. Individuals on any medications, or those with a history of hypertension, diabetes or hypercholesterolaemia were excluded to reduce the effect of any confounding atherosclerotic risk factors. All subjects fasted (including no smoking for 24 hours) before having blood pressure, height and weight measurements taken. White cell, monocyte, platelet count and MPV were measured using a Coulter Stacks S Blood analyser by Dr. RK Singh.

4.2.3 Terminal restriction fragment analysis

DNA extraction and terminal restriction fragment analysis was performed as described in Chapter 2.

4.2.4 Statistical analysis

Characteristics of cases and controls were compared using unpaired t-test for continuous variables and chi-squared test for categorical variables. All analysis was carried out in Stata Statistical Software (Release 7.0; Stata Corporation, College Station TX; 2001). Advice and assistance was provided by Professor JR Thompson.

4.3 RESULTS

4.3.1 Demographics

A total of 24 male subjects with a successive two generational history of premature MI were identified from 23 different families. The 24 controls were matched for both age and smoking.

Table 4.1: Demographics of cases and controls

Demographic	*Cases (n = 24)*	*Controls (n = 24)*	*p-value*
Age at interview (years)	27.1 ± 6.4	26.1 ± 6.1	0.6
Blood pressure (mmHg)			
Systolic	131.5 ± 12.7	123.7 ± 8.5	0.0178
Diastolic	82.7 ± 10.2	72.4 ± 8.1	0.0004
Body Mass Index (kg/m^2)	25.8 ± 4.6	23.9 ± 3.7	0.1333
WBC count (x $10^3/\mu l$)	6.6 ± 1.6	5.9 ± 1.2	0.1181
Monocyte count (x $10^3/\mu l$)	0.5 ± 0.2	0.4 ± 0.1	0.1472
Platelet count (x $10^3/\mu l$)	242.3 ± 61.9	252.2 ± 58.3	0.5782
MPV (fl)	8.8 ± 1.1	8.9 ± 1.0	0.9533
Current/non-smokers	7/17	8/16	0.9881
Hypertension[a]	n = 0	n = 0	n/a
Diabetes mellitus[b]	n = 0	n = 0	n/a
Hyperlipidaemia[c]	n = 0	n = 0	n/a

Data are shown as mean ± standard deviation. [a] Defined as systolic BP ≥ 140mmHg or diastolic BP ≥ 90mmHg, or the use of antihypertensive medication. [b] Defined as glucose concentration ≥ 6.0 nmol/l or the use of anti-hyperglycaemic medication. [c] Defined as LDL-cholesterol ≥ 160mg/L or the use of lipid lowering therapy.

Cases and controls were very well matched for age and haematological parameters; platelet counts, MPV and smoking status all showed non-significant differences. There was also no evidence of hypertension, diabetes mellitus or hyperlipidaemia in either cases or controls (for definitions, see Table 4.1). Systolic and diastolic BPs were, however, significantly higher in the offspring of cases compared to controls.

4.3.2 Distribution of mean TRF lengths

The distribution of mean white cell TRF length is shown for both cases and controls in Figure 4.1. As in Chapter 3 there was a high degree of inter-individual variation in mean TRF length in both groups.

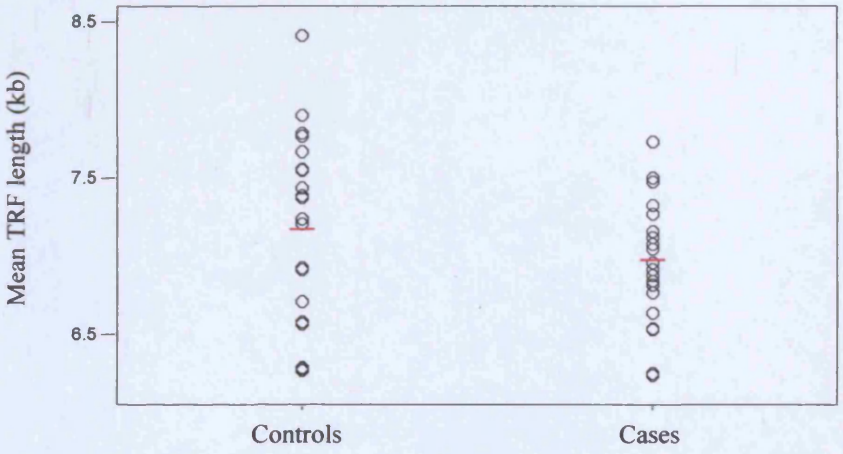


Figure 4.1: Boxplot of mean TRF length for offspring of cases and offspring of controls. The mean TRF of offspring of cases is slightly smaller than that of controls.

Despite the small sample sizes, and the relatively young age of the cohort, there was a 240bp difference in length between cases and controls. However, after adjustment for age this did not reach statistical significance (p=0.118).

4.3.3 Correlation in mean TRF lengths between parents and offspring

As I had estimates of mean TRF length for a selection of both the parents and their offspring I was able to examine the correlation in length between the two groups. I was able to pair 13 offspring of cases to their parents, and 9 controls. The correlation in mean TRF length between parents and offspring is shown in Figure 4.2.

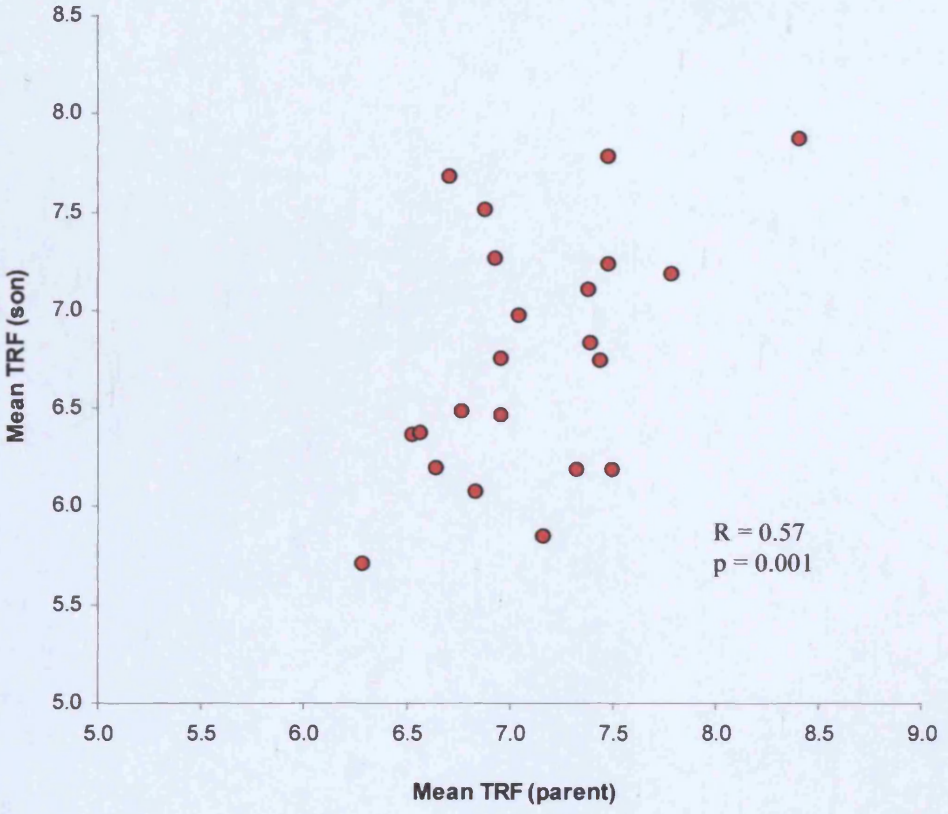


Figure 4.2: Correlation of mean TRF lengths between parent and offspring.

Figure 4.2, demonstrates a strong correlation in age-adjusted mean TRF length between parents and their offspring (R = 0.57, p= 0.001).

4.4 DISCUSSION

This study showed that the mean TRF length of WBCs is approximately 240bp shorter in the healthy male offspring of patients with CHD, compared to healthy male offspring with no family history of CHD. While this difference was not statistically significant, in light of the 300bp difference demonstrated between cases and controls (that is, between the parents) in the previous study, it is a very interesting observation. This is compounded by the significant correlation in mean TRF length between parents and offspring in this study, regardless of the clinical status of the parents.

4.4.1 Possible reasons for the observed difference in mean TRF length between offspring of cases and offspring of controls

As in the previous study there a number of possible explanations for the difference in mean TRF length between the groups.

The first is that the difference is a chance finding, and this is supported by the lack of statistical significance. However, the small sample size in this study, compared to that described in Chapter 3, may also explain why the difference does not reach statistical significance.

The second is that both sets of offspring inherited telomeres of a similar length, but offspring of cases with premature CHD have experienced a greater rate of telomere attrition. Over time this could result in a divergence in length and so I assessed the effect of a number of relevant factors. By design, all subjects recruited for the study were healthy with no evidence of diabetes mellitus, hyperlipidaemia, or hypertension as defined in table 4.1. While neither group exceeded these values, the offspring of cases showed significantly higher systolic ($p=0.0178$) and diastolic ($p=0.0004$) BP values than controls. This is an interesting observation, given that a history of hypertension was associated with shorter telomeres in Chapter 3. There were no significant differences in BMI, smoking status or haematological parameters including WBC, monocyte and platelet counts, or MPV. Of course, there is the possibility that an unmeasured factor has affected the rate of attrition. In dissecting the results of Chapter 3 I discussed the role of the telomere-binding protein, TRF2, in disruption of the telomeric complex, resulting in atherogenic changes to the endothelium (Minamino *et al.*, 2002). There is the possibility that variants of such proteins exist that result in a less stable telomere, resulting in accelerated attrition. Perhaps the reason I have only demonstrated a small, non-significant difference in mean TRF length between offspring of cases and controls is because they inherited telomeres of similar length, but that offspring of cases have inherited variants of the binding proteins that resulted in increased attrition, thus, the difference in length would be amplified over time. It would be interesting to follow these subjects for 20-30 years to see if the two groups partition such that we see a significant difference in later life. Indeed, by the age of 50 we may see the 300bp difference reported in both the pilot study (Samani *et al.*, 2001), and in Chapter 3.0. However, this explanation would ***not*** be consistent with the hypothesis that telomere length in inherited.

The third explanation for the observed difference is that the offspring of subjects with premature CHD have inherited shorter telomeres, and this is discussed below.

4.4.2 Correlation in TRF lengths between parents and offspring

In this study I was able to pair the mean TRF data of 22 offspring to their parents and examine the correlation between the two. Even with these relatively small numbers I found a strong correlation in mean TRF length ($R = 0.57$, $p = 0.001$). This observation lends support to the hypothesis that telomere length is, at least in part, a heritable trait. In addition, the shorter mean TRF lengths in subjects with a two-generational history of premature CHD may help explain the increased risk associated with a positive family history.

4.4.3 Limitations of the study

The small sample size in this study may explain the non-significant difference in length between the two groups. This issue could be addressed using a much larger study. Even if there is a true difference present, it may be that it is smaller than the 300bp difference observed in Chapter 3: Figure 3.2 showed a slight divergence in the trend lines, thus the difference may be smaller at a young age. In Chapter 3 I demonstrated a 300bp difference between cases and controls, with a mean age of 47 years. The rates of attrition for cases and controls were 28.3 and 24.8bp/year, respectively. Using these rates of attrition, regression to 26.6 years (the average age of the offspring in the present study) results in a difference of 207bp. A smaller difference in length in the offspring would be harder to detect: a power calculation shows that to detect a difference of 200bp at the 0.05 level we would need to have groups of 130 to achieve 80% power, and 172 to achieve 90% power.

In summary, although the offspring of subjects with premature MI show no clinical signs of CHD, they do have slightly shorter telomeres than controls. This, coupled with the associations previously demonstrated between shorter telomeres and risk of MI (Chapter 3), and Cawthon *et al.* have demonstrated between shorter telomere length and increased mortality rate from heart disease (Cawthon *et al.*, 2003), indicate that they may be at an increased risk of cardiovascular disease. The heritiablity of telomere length is also supported by the strong correlation in telomere lengths between parents and

offspring. If there were a genetic tendency to shorter telomeres in individuals with a two generational history of premature MI, I would expect to observe the difference from birth. The fact that I have only observed a small, non-significant difference in this study is possibly due to the small cohort size. It could also be because the difference in telomere length will be exacerbated over time. The fact that there is any difference at all is interesting as it alludes to the possibility that there is a familial element to CHD that has hitherto been unexplained. Clearly, these observations need to be extended, using a much larger cohort.

A PROSPECTIVE STUDY OF THE ASSOCIATION BETWEEN TELOMERE LENGTH AND CVD

5.1 INTRODUCTION

Chapter 3 demonstrated a significant association between shorter telomeres and risk of premature MI, supporting the association observed within our group in a study (Samani *et al.*, 2001) of 10 patients with severe triple-vessel disease and 20 age and sex-matched controls. While the study demonstrated a graded relationship between progressively shorter telomeres and increased risk of MI, the cases represent the *survivors* of the event. Up to 30% of subjects who suffer an MI do not survive the acute event, thus I am unable to exclude the possibility that possessing longer telomeres adversely affects the immediate prognosis after MI. To address this issue it was necessary to carry out a large, *prospective* study.

A high level of LDL-cholesterol is an important risk factor for CHD. One of the therapeutic approaches available is to inhibit the endogenous synthesis of cholesterol by blocking the irreversible conversion of 3-hydroxy-3-methyl-glutaryl-CoA (HMG CoA) to mevalonic acid, the rate-limiting step, by the enzyme HMG CoA reductase. The West of Scotland Coronary Prevention Study (WOSCOPS) was a prospective, double-blind primary prevention study of approximately 6000 middle-aged men, randomly assigned to receive either a HMG CoA reductase inhibitor (pravastatin) or placebo. The clinical progress of the subjects was then recorded over a period of 5 years in order to evaluate the effectiveness of a HMG CoA reductase inhibitor in preventing coronary events in men with moderate hypercholesterolemia and no history of myocardial infarction. The study found that, compared with placebo, pravastatin reduced the risk of fatal or non-fatal coronary events by approximately 30% with no evidence of major adverse effects or increase in death from non-cardiovascular causes (Shepherd *et al.*, 1995).

The WOSCOPS investigators had the foresight to create a biobank of DNA and plasma collected at baseline to allow subsequent investigation of potential risk factors with risk of CHD using a nested case-control approach.

In this chapter I examined the association of baseline mean telomere length with risk of subsequent CHD in this well-characterised cohort, measured using a real-time PCR-based approach. The aim was to investigate potential difference in baseline mean

telomere length between those subjects who experienced a clinical event, and matched controls without events, and to examine any interaction with statin treatment.

5.2 METHODS

5.2.1 Subjects

The WOSCOPS study has been described in detail in a number of publications explaining the design and its clinical outcomes (Shepherd *et al.*, 1995; Shepherd, 1995). The baseline characteristics of the WOSCOPS participants have been detailed previously (The WOSCOPS Study Group, 1995). The WOSCOPS cohort comprised 6595 males age 45-65 (mean 55.2 years) with mean baseline total cholesterol of 7.0mmol/l, HDL cholesterol of 1.2mmol/l and mean triglycerides of 1.8mmol/l. Recruits had no history of myocardial infarction and had normal renal and hepatic function. For the nested case-control studies, subjects experiencing a definite or suspect fatal or non-fatal myocardial infarction, sudden coronary death, or required coronary artery bypass graft or angioplasty during the course of the study were defined as cases (n=580). Each patient was matched to two controls, selected from the original cohort of 6595, to give a total of 1160 controls, on the basis of age (using 2-year age categories) and smoking status. Of the 580 cases, 507 cases had "hard" end-points (death or MI).

5.2.2 Plasma lipids

Plasma lipids and lipoproteins were measured in fasting plasma samples according to the protocols of the Lipid Research Clinics (Lipid Research Clinics Program, 2004) LDL peak particle diameter, reported as the particle diameter of the major LDL fraction, was used as an index of LDL size distribution and was determined as described previously (Friedlander *et al.*, 2000) with the exception that 2–10% polyacrylamide gel electrophoresis was used.

Subject recruitment and subsequent baseline measurements were carried out by the WOSCOPS investigators.

5.2.3 PCR Analysis

Working plates were prepared at 5ng/ul, and all samples were subjected to real-time PCR analysis, as described in Chapter 2.3. Samples were amplified in duplicate within

each PCR run. PCR analysis was carried out in a blinded fashion, and analysed using Comparative Quantification (Rotogene 6.0.1, Corbett Research, Cambridge, UK).

5.2.4 Statistical methods

Differences in baseline characteristics were assessed using conditional logistic regression, and differences between cases and controls were compared using a *t*-test or Wilcoxon test using the SAS statistical package, (version 8.02). Differences in baseline mean telomere length and the effect of both age and statin treatment were carried out using Stata Statistical Software (Release 7.0; Stata Corporation, College Station TX; 2001). Advice and assistance was provided by Professor JR Thompson.

5.3 RESULTS

Of the 580 subjects defined as cases, and 1160 controls, only 498 and 1108 were available in the WOSCOPS biobank. Of these, a small number did not amplify during the PCR assay (14 cases, 50 controls), resulting in final numbers of 484 cases and 1058 controls.

5.3.1 Demographics

The baseline characteristics of the subjects are shown in table 5.1.

Table 5.1: Baseline characteristics of case and control groups*

Characteristic	*Cases (n = 484)*	*Controls (n = 1058)*	*p-value*
Age (years)	56.9 (5.1)	56.7 (5.2)	a
Body mass index (kg/m^2)	26.0 (3.2)	25.6 (3.2)	0.04
Smokers (%)[a]	266 (53%)	606 (55%)	a
Alcohol consumption (units/week)	11 (13)	11 (13)	0.55
Total cholesterol (nmol/l)	7.08 (0.61)	7.02 (0.57)	0.09
Triglycerides (nmol/l)	1.96 (0.83)	1.84 (0.77)	0.05
LDL cholesterol (nmol/l)	5.02 (0.46)	4.95 (0.44)	0.04
HDL cholesterol (nmol/l)	1.07 (0.22)	1.14 (0.25)	<0.001
LDL diameter (nm)	26.33 (0.85)	26.40 (0.89)	0.17

a Cases and controls were matched for age and smoking. Data given for continuous variables are mean (standard deviation), and for categorical variables are the number of subjects (%).

Cases and controls were matched for both age and smoking. Alcohol consumption and total cholesterol did not differ significantly. Triclycerides and LDL cholesterol were significantly higher in cases, while HDL cholesterol was significantly higher in controls. LDL particle diameter did not differ between the two groups.

5.3.2 Real time telomere PCR analysis

Figure 5.1 illustrates the raw PCR data, with the Comparative Quantification (CQ) Ratio plotted as a function of age.

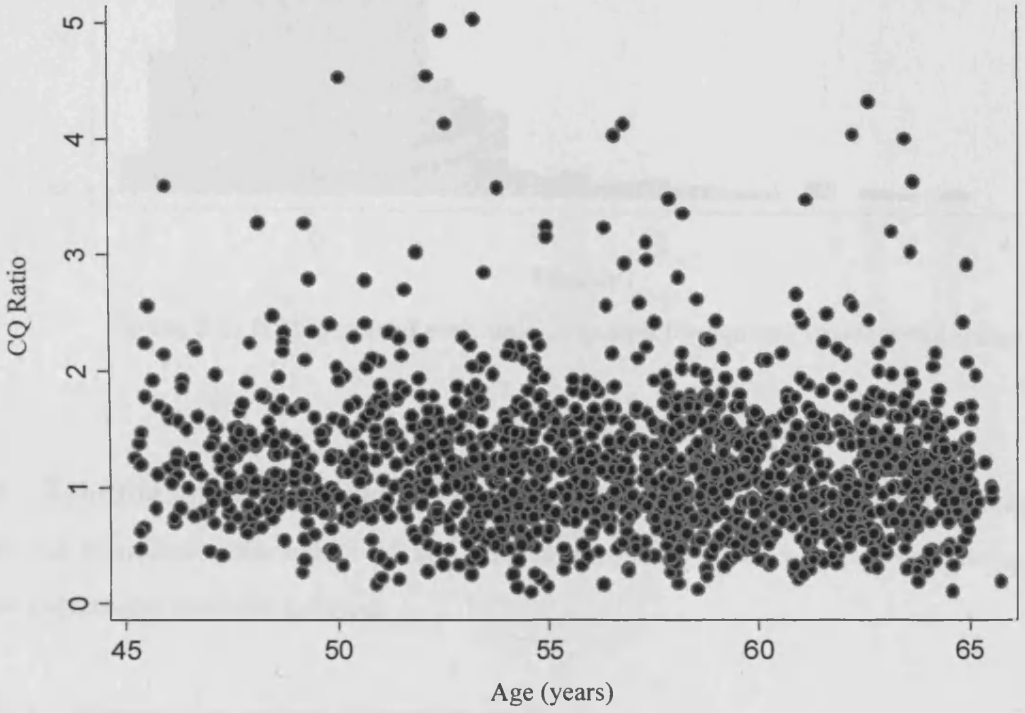


Figure 5.1: CQ Ratio v Age. Note the wide degree of scatter, and the presence of several samples giving very high ratios.

Because of the non-normal distribution of the data, robust regression analysis was used to down-weight the effect of outliers. Log-transformation was considered, but felt to be less appropriate as examination of the residuals (Figure 5.2) showed the data was still non-normally distributed.

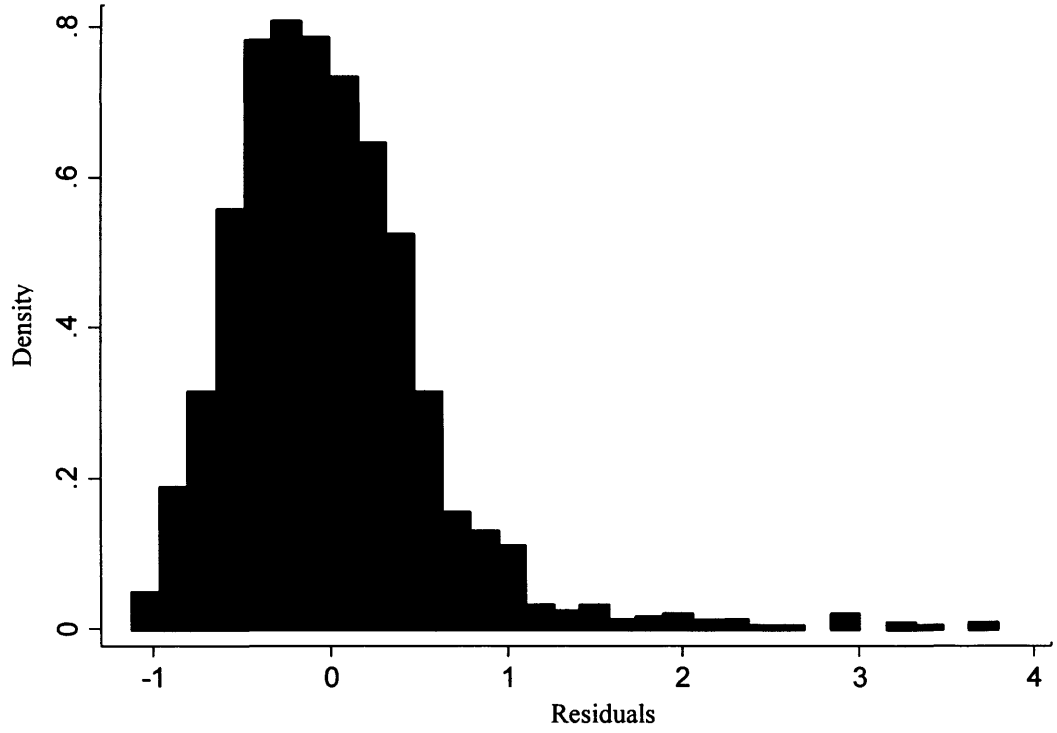


Figure 5.2: Histogram of residuals. Adjusted for age and case/control status.

5.3.3 Relationship of age and cases status on baseline mean telomere length

Table 5.2 summaries the effect of age and case-control status on CQ ratio using the robust regression analysis method.

Table 5.2 Summary of robust regression analysis

Subjects	*Effect of*	*Effect on CQ Ratio*	*p-value*
Controls & all end-points	Age	-0.008 ± 0.002	0.001
	Case Status	-0.075 ± 0.027	0.005
Controls & hard end-points only	Age	-0.008 ± 0.002	0.001
	Case Status	-0.074 ± 0.029	0.010
Controls & deaths only	Age	-0.008 ± 0.002	0.002
	Case Status	-0.070 ± 0.050	0.161

Age had a highly significant effect on CQ ratio with a reduction in the ratio of –0.008 ± 0.002 per additional year of age. After adjusting for age, status also had significant effects for all end-points, as well as hard end-points, with cases having a CQ ratio of

approximately 0.075 ± 0.002 lower than controls. The trend was very similar for deaths but, because of small numbers, did not reach significance. Therefore, mirroring data shown in Chapter 3, mean telomere length at baseline in those who became cases was similar to controls subjects approximately 9.4 years older.

5.3.4 Telomere length, risk of CHD and benefits from statin treatment

Subjects in the WOSCOPS study were randomised to receive either pravastatin, or placebo. This allowed an assessment of any interaction between baseline telomere length and statin treatment on risk of subsequent CHD. Tables 5.3 and 5.4 separately summarise the number and percentage of placebo and pravastatin-treated subjects who suffered an event in each tertile of CQ ratio, for all end-points, and hard end-points, respectively.

Table 5.3: Percentage of subjects per tertile suffering an event (all end-points)

Tertile of CQ Ratio		*No Treatment*	*Treatment*
T1) 1.327 - 5.043	Subjects	262	252
	Events	67	65
		25.57%	**25.79%**
T2) 0.902 - 1.326	Subjects	279	235
	Events	115	66
		41.22%	**28.09%**
T3) 0.00 - 0.901	Subjects	265	249
	Events	107	64
		40.38%	**25.70%**

In the untreated group there were significantly fewer events in tertile 1 (T1, longest telomeres), compared to T2 and T3, with no difference observed between T2 and T3. In treated subjects the number of events in each tertile was similar with each other, and also similar to the number in the longest telomere tertile with no treatment.

Table 5.4: Percentage of subjects per tertile suffering an event (hard end-points)

Tertile of CQ Ratio		*No Treatment*	*Treatment*
T1) 1.327 - 5.043	Subjects	262	252
	Events	52	20
		19.85%	**19.84%**
T2) 0.902 - 1.326	Subjects	279	235
	Events	101	59
		36.20%	**25.11%**
T3) 0.00- 0.901	Subjects	265	249
	Events	98	55
		36.98%	**22.09%**

The pattern with hard end-points followed a very similar pattern to all end-points (Table 5.4). The interaction between telomere length and treatment on event was highly significant for all end-points (p=0.0047) and hard end-points (p=0.0044).

5.4 DISCUSSION

In this chapter, real-time PCR was used to analyse the effect of telomere length on risk of CHD in a large, prospective study. The design of the study matched 2 controls to each case to give a total of 580 cases and 1160 controls. A number of samples were not available from the biobank, or failed to amplify in the assay, resulting in final numbers of 484 cases and 1058 controls.

There was a large degree of scatter in measured CQ ratios at all ages, and a considerable number of outliers with the potential to significantly skew the data. In such cases the data need to be manipulated such that the outliers are down-weighted: the method used depends on whether the outliers are extremes, or erroneous. Using log-transformation on the PCR data would imply that telomere length changes as a percentage of the total length, rather than at a constant rate. However, the results of Chapter 3 and current reports in the literature suggest that the rate of attrition in adulthood is relatively constant (Allsopp *et al.*, 1992; Samani *et al.*, 2001), compared to childhood (Zeichner *et al.*, 1999). In addition, for log-transformation to be valid the transformed data should be normally distributed, but the plot of residuals (Figure 5.2) showed that this was not the case. The alternative, robust regression analysis, uses the median rather than the mean value of CQ ratio at any given age for the regression, giving less weight to the outliers. This type of analysis is particularly useful when it is believed that the outliers represent

errors, rather than extremes, of measurement. This study examined a ratio obtained from a pair of PCR reaction. PCR is a multiplicative process and it is conceivable that, in those individuals with extremely long telomeres, the PCR cannot accurately measure the length. Perhaps the amplification in these few cases results in an amount of product that is outside the dynamic range of the assay. The observation that telomeres appear to erode at a constant, rather than a logarithmic, rate, and the failure of log-transformation to give normally distributed data, indicated that robust regression analysis was appropriate. This method was then used to demonstrate a significant age-related effect ($p = 0.001$), and a significant difference in telomere length between controls and all end-points ($p = 0.005$) and hard end-points only ($p = 0.01$). Given the difference in CQ ratio between the two groups (0.075), and the age-effect observed (0.008/year), telomere length in cases is similar to controls approximately 9.4 years older. This value is in remarkable agreement with that reported by Samani *et al.* (Samani *et al.*, 2001) (8.6 years), and in Chapter 3 (11.4 years).

These findings, from a prospective study, associate baseline mean telomere length with risk of CHD. They also support and extend those of Cawthon *et al.* (Cawthon *et al.*, 2003), and strongly suggest that telomere length is aetiologically linked to risk of CHD.

5.4.1 Telomere length and benefits from stain treatment

The range of telomere lengths was divided into tertiles (T) to allow examination of the percentage of subjects, separated into those with and without statin treatment, who experienced a clinical event. When considering all end-points **not** receiving treatment, there was a significant increase in the percentage of subjects experiencing an event as telomere length decreased from T1 (25.6%) to T2 (41.2%), but no difference between T2 and T3 (40.4%). In those subjects receiving treatment the percentage did not differ significantly from T1 to T3 (25.8%, 28.1% and 25.7%). The results for hard end-points followed a very similar trend, with those **not** receiving treatment experiencing an increased percentage of events from T1 (19.6%) to T2 (36.2%), and no difference between T2 and T3 (37%). In those subjects receiving treatment there was only a slightly increased percentage of events in T2 (25.1%) and T3 (22.1%) compared to T1 (19.8%).

While the number of events in untreated subjects increased from T1 to T2 in the present study, there was no increase as telomeres shortened further. This was a slightly

surprising result, given that in Chapter 3 a graded relationship was observed between shorter telomeres and an increased risk of premature MI. One possible explanation is that a number of different phenotypes were examined in this study, but only premature MI was examined in Chapter 3. An alternative explanation is that subjects recruited for the WOSCOPS study were at an increased risk of CHD at baseline: Scotland has the highest age-standardised death rate from CHD per 100,000 population in the entire UK (British Heart Foundation, 2003). More specifically, all subjects recruited had a baseline total cholesterol level of above 7 nmol/l; the mean blood cholesterol level in men in England is 5.5nmol/l (British Heart Foundation, 2003), and the National Service Framework for CHD suggests a cholesterol target of 5.0nmol/l (Department of Health, 2000).

If the primary hypothesis is correct, and inheriting shorter telomeres results in increased risk of CHD, the relatively high incidence of CHD in Scotland may reflect shorter telomeres in the region as a whole, a situation exacerbated by the elevated serum cholesterol levels observed. There will be a threshold of telomere length, after which a clinical event is very likely, and it may that all subjects in the present study were already very close to it. The subjects in Chapter 3 may have had a mean age adjusted telomere length that is greater than that in the present study, thus a graded relationship was observed. In this study the threshold may have been reached sooner (in T2) as the overall telomere length of the subjects was shorter, thus only those individuals with the very longest telomeres were afforded any degree of protection. This theory is supported to some extent by the results of Chapter 3. In Figure 3.3 the risk of MI increased significantly from quartile 1 (Q1) to Q2, and from Q2 to Q3, but there was only a very small increase in risk from Q3 to Q4 (the shortest telomeres), suggesting that the threshold was being reached in the very shortest quartile.

Whether considering all end-points, or hard end-points only, the percentage of **treated** subjects suffering an event did not increase as telomeres shortened, and in T2 and T3 event rates were similar to **untreated** subjects with the longest telomeres. These data indicate that treatment with a statin is associated with a reduction in the number of clinical events, only in those subjects with increased risk based on their telomere length. These are highly novel and provocative findings, which require further explanation.

The development of atherosclerosis is thought to be initiated by endothelial damage (Ross, 1999b) due to the presence of various cardiovascular risk factors, thus the

reduction in the number of events in statin-treated subjects indicate that statins may *directly* influence endothelial function. While repair of endothelial damage was originally thought to result from migration and proliferation of endothelial cells (ECs) from viable endothelium adjacent to the site of injury, recent evidence suggests an alternative mechanism. Studies in both animals (Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001) and human subjects with stable CAD (Vasa *et al.*, 2001a) have demonstrated that statins may mobilise bone marrow-derived endothelial progenitor cells (EPCs), resulting in accelerated endothelial repair (Walter *et al.*, 2002). The increase in EPC number has been shown to plateau at a 3-fold increase after 3 to 4 weeks of statin therapy (Vasa *et al.*, 2001a).

Damage to the endothelium results in increased EC turnover with a concomitant decrease in telomere length. Once the telomere reaches a critical length the cell enters senescence and can no longer divide. In the absence of an alternative mechanism for endothelial repair, the endothelium is unable to cope with continued cellular insult and complications of atherosclerosis may occur. An increase in the number of EPCs provides such a mechanism but, interestingly, EPCs isolated from patients with CAD had previously revealed an impaired migratory response, which was inversely correlated with the number of risk factors (Vasa *et al.*, 2001b). Thus, not only do risk factors result in damage to the endothelium, they also impair the function of the EPCs that would be able to counteract the damage. In relation to the present study, those subjects with the shortest telomeres may reach a state of endothelial dysfunction earlier than those with longer telomeres, resulting in atherogenic changes to the endothelium, and an increase in clinical events (as seen in the untreated group). This would have been exacerbated by the presence of risk factors that impair EPC function. Treatment with a statin may have provided protection against the initiation of atherogenic changes by increasing the number of circulating EPCs, allowing the endothelium to cope with a degree of insult that would otherwise lead to endothelial dysfunction. While this may provide accelerated endothelial repair, it may also result in accelerated telomere attrition in the EPCs, such that they reach senescence earlier. However, it has recently been shown that EPCs demonstrate telomerase activity and, interestingly, oxLDL reduces the activity of the enzyme by approximately 50%, an effect that was significantly abolished by pretreatment with atorvastatin (Imanishi *et al.*, 2004). These results suggest elevated LDL not only inhibits the migratory response of EPCs, but once oxidised can also

inhibit telomerase activity in EPCs, resulting in premature senescence of these cells. Thus, in addition to the direct inhibition of cholesterol synthesis, statins indirectly reduce the levels of oxLDL, abrogating the reduction in EPC telomerase activity.

In summary, this large, prospective study demonstrated that subjects experiencing CHD had significantly ($p = 0.005$) shorter telomeres than age and sex-matched controls at baseline. In biological terms, cases were the same age as controls over 9 years older, at least as far the telomeres of their WBCs were concerned. This supported previous observations of an 8.6 year difference between controls and subjects with severe triple vessel disease (Samani *et al.*, 2001), and an 11 year difference between controls and subjects experiencing a premature CHD (Chapter 3). In addition, treatment with a cholesterol-lowering drug (statin) ameliorated the increased number of clinical events in subjects with progressively shorter telomeres. Statin treatment in young, healthy subjects with no clinical evidence of cardiovascular disease may protect against the development of atherosclerosis through a number of effects: directly, through inhibition of cholesterol synthesis, and indirectly by increasing the number of circulating bone marrow-derived EPCs. In addition, statins reduce the susceptibility of lipids to oxidation, thus reducing the levels of oxLDL that can inhibit EPC telomerase activity.

MAPPING OF A LOCUS DETERMINING TELOMERE LENGTH IN HUMANS

6.1 INTRODUCTION

The preceding chapters have demonstrated a strong association between a shorter mean TRF length in WBCs and increased risk of premature MI, showed that the offspring of patients with premature CHD have shorter (albeit non-significant) telomeres than age and sex matched controls, and demonstrated a partitioning in mean WBC telomere length between those who develop CHD and those who do not in a prospective study. Furthermore, a significant correlation in telomere length between parents and children has been demonstrated. This observation, combined with reports in the literature (Jeanclos *et al.*, 2000; Nawrot *et al.*, 2004; Slagboom *et al.*, 1994), indicate that there is a strong genetic determination of the length, or regulation of the length, of telomeres. Thus, the purpose of the work in this chapter was to see if a locus or loci determining telomere length could be mapped, using Quantitative Trait Loci (QTL) Linkage Analysis.

6.1.1 QTL Linkage Analysis

Some disorders have a simple Mendelian mode of transmission, where a specific mutation results in development of the disorder. The general strategy to identify the genes for such traits is called 'classical linkage' and is based on Fisher's theory of likelihood inference (Fisher, 1918). Classical linkage models the distance between a DNA marker locus and a putative disease locus in a small number of large, multigenerational pedigrees consisting of both affected and unaffected family members. More complex diseases are multi-factorial, being influenced by different genes and their interaction with various environmental factors. Quantitative traits are influenced by the developmental interplay of various genes and the environment, with each of the genes that exerts an influence being referred to as a *polygene*. The locus of such a polygene is called a quantitative trait locus (QTL). To detect QTLs, *non-parametric* (model-free) linkage analysis is performed: several hundred DNA markers are examined in siblings (and their parents wherever possible), and allele sharing between siblings (or other relatives) is investigated. It is important to note that there are two definitions of allele-sharing:

1) *identity-by-state* (IBS) – two alleles of the same form (identical sequence);
2) *identity-by-descent* (IBD) – if, in addition to being IBS, two alleles are descended from the same parental gene.

Thus, linkage of a marker to a QTL implies that the differences in the trait between the relative pairs will be smaller if they share the same version of the marker, obtained from the same parent (Haseman and Elston, 1972), as illustrated in Figure 6.1.

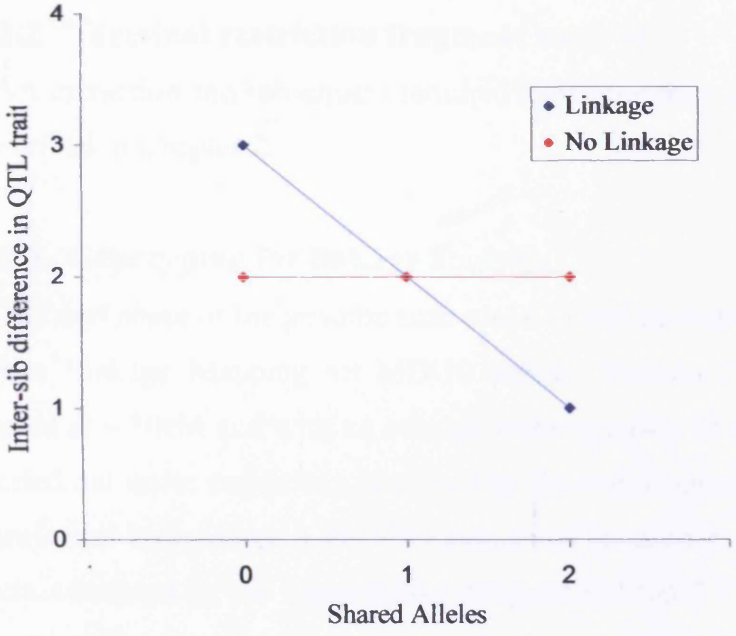


Figure 6.1: QTL Linkage. If the marker is not linked to the QTL, the difference in the telomere length between siblings will not differ significantly, regardless of the number of shared alleles (red line). However, if the marker *is* in linkage, the difference in telomere length between siblings will decrease as they share more alleles (blue line).

Thus, the aim of this chapter was to see if a locus (or loci) affecting telomere length in humans could be mapped by performing a QTL analysis of mean leucocyte telomere restriction fragments, as measured by Southern blotting, in a large cohort of sib-pairs.

6.2 METHODS

6.2.1 Subjects

Families were selected from those participating in the British Heart Foundation (BHF) Family Heart Study. This is a large study two-centre study (Leicester and Leeds) of over 2000 Caucasian families recruited from throughout the United Kingdom whose primary objective is to map loci predisposing to premature CHD. Families for the telomere length analysis were chosen randomly from those recruited in the Leicester centre.

6.2.2 Terminal restriction fragment analysis

DNA extraction and subsequent terminal restriction fragment analysis was performed as described in Chapter 2.

6.2.3 Genotyping for linkage analysis

In the first phase of the genome scan a total of 400 microsatellite markers from the ABI-Prism Linkage Mapping set MD-10 panels (Version 2.5: PE Applied Biosystems) spaced at ~ 10cM and with an average heterozygosity of 0.79 were analysed. PCR was carried out under conditions described by the manufacturers and the products pooled in panels and analysed on a PE 3700 automated sequencer. Genotypes from each marker were examined by the GeneMapper program version 2.0 (PE Applied Biosystems). In the second phase, an additional 4 markers were analysed to refine the chromosome 12 linkage region. Two markers (D12S1640, D12S1663) were selected from ABI-Prism Linkage Mapping set MD-5 panels (Version 2.5: PE Applied Biosystems) and 2 markers (D12S1698, D12S1589) were selected from the NCBI database.

6.2.4 Quality control of genotype data

All genotypes, including those that passed GeneMapper's internal quality control, were manually read by at least one individual (i.e. of those working on the Family Heart Study) and those genotypes that did not fully pass the program's quality control were read by two independent individuals. Genotypes were rejected and the samples re-run unless there was complete agreement on allele calls. RELATIVE (Goring and Ott, 1977) and GRR (Abecasis *et al.*, 2000) programs were used to confirm family relationships. Inheritance within families was verified using the Pedcheck program

(O'Connell and Weeks, 1988). If there was a likelihood of inheritance errors, the complete family was genotyped again for that marker.

6.2.5 Statistical analysis

Summary statistics on the pattern of change in mean TRF length with age in men and women were obtained from random effects regressions allowing for correlation within families.

Two-point and multipoint quantitative trait linkage analyses were conducted by using Sequential Oligogenic Linkage Analysis Routines (SOLAR) package (version 1.7.3) (Almasy and Blangero, 1998). Files were converted to SOLAR format using MEGA2 (version 2.5R2) (Mukhopadhyay *et al.*, 1999). Allele frequencies were calculated from the observed genotypes. The order of the markers loci and their recombination distances used for multipoint linkage analysis were based on the DECode map (Kong *et al.*, 2002) supplemented with data from NCBI.

Linkage of variance component was assessed by fitting a polygenic model that does not incorporate genetic marker information and comparing it with models that incorporate genotype data at a specific marker (2-point analysis) or across a chromosome (multipoint analysis). To adjust for age and sex, these factors were initially included as covariates in the linkage analysis. Age and sex were found to be significant and were retained in the linkage-analysis model. Heritability values were obtained after adjustment for covariates. The data were also analysed using the variance component function in the Multipoint Engine for Rapid Likelihood INference (MERLIN) program (Abecasis *et al.*, 2002) after prior adjustment for age and sex. Simulations were conducted in MERLIN using the same conditions of the main QTL analyses, and using the option –simulate.

Heritability is referred to as a portion of the phenotypic variation among individuals that is due to genetic differences among them, thus it is defined as the ratio of genotypic variance to the phenotypic variance.

Analysis was performed with the assistance of Dr. M Mangino and Professor JR Thompson.

6.3 RESULTS

6.3.1 Subject details

In total 383 adult subjects from 173 families comprising 258 sib-pairs were analysed (291 males and 92 females).

Table 6.1: Summary of sibling pairs studied.

Individual statistics	*n*
Total	383
Males	291
Females	92
Family statistics	
Total families	173
2 pair	141
3 pair	25
4 pair	5
5 pair	2
Sib-pair statistics	
Total	258
Male-male	154
Female-female	24
Mixed	80

The mean age of the subjects was 65.8± 6.4(sd) years (range 47 to 82 years).

6.3.2 Distribution of mean TRF lengths

As in the Chapter 3, there was a wide scatter in mean TRF lengths at any given age, and an age-related decrease in mean TRF length in both men (29.9 ± 5.6 bp/yr) and women (16.8 ± 9.9 bp/yr) (Figure 6.1).

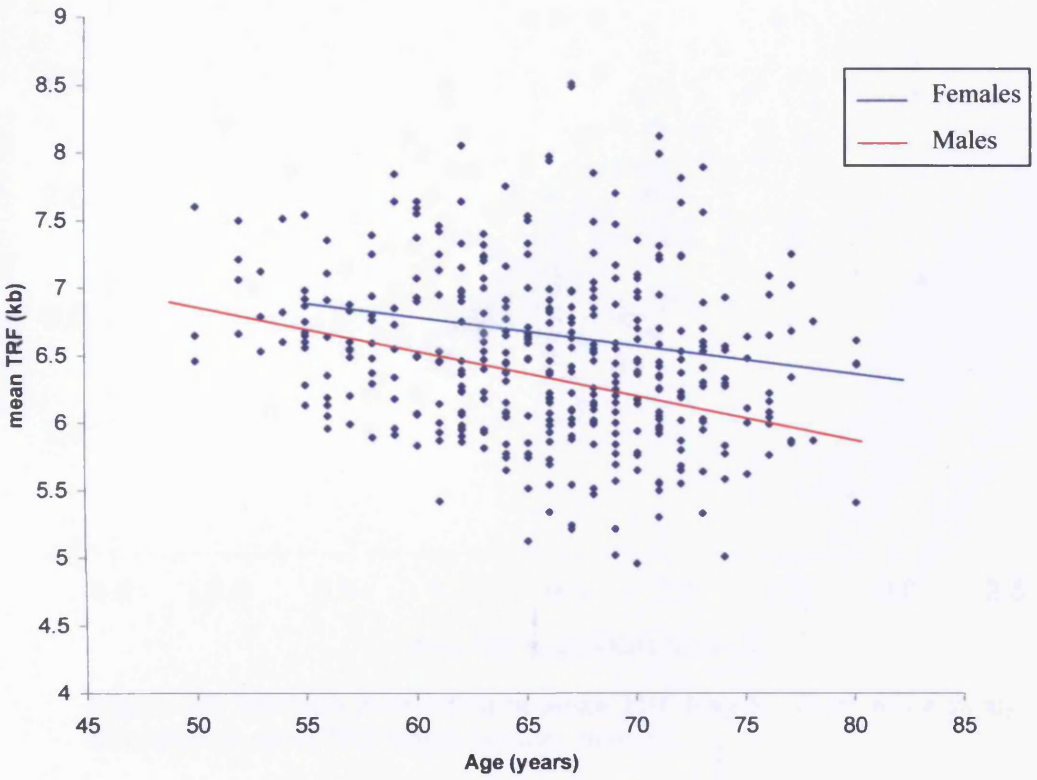


Figure 6.1: Mean TRFs for male and females. Note the high inter-individual variability in mean TRF length at any age. The regression lines show the decrease in meanTRF length with age in females (blue lines) and males (red line).

The difference in rate of decline between the sexes was not significant (p=0.61). However, the mean age-adjusted TRF length in men was shorter than in women (difference, 271.5±64.7(se) bp, p<0.001).

6.3.3 Inter-sibling correlations

Figure 6.2 shows the highly significant inter-sibling correlation in mean TRF length; a heritability index (h2) of 81.9±11.8% was obtained.

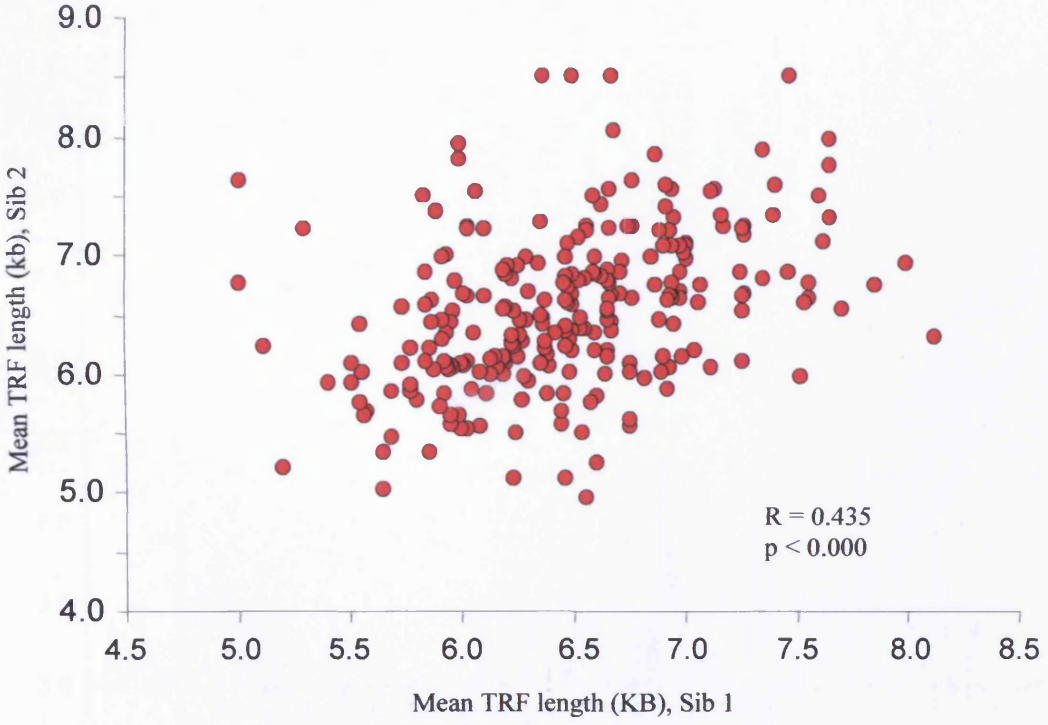


Figure 6.2: Inter-sib correlation in mean TRF length. There was a strong correlation in mean TRF length between siblings.

Age ($p < 0.001$) and sex ($p < 0.001$) were significant co-variates, together accounting for 8.7% of the variance in the trait, and were included in the linkage-analysis model to allow appropriate adjustment.

6.3.4 Genome-wide scan

An initial genome scan with 400 microsatellite markers at intervals of ~10cM (ABI-Prism Linkage Mapping set MD-10 panels version 2.5) with an average heterozygosity of 0.79, identified significant linkage to chromosome 12 (Figure 6.3). A maximum two point LOD score of 3.21 was obtained for the marker D12S345 using SOLAR.

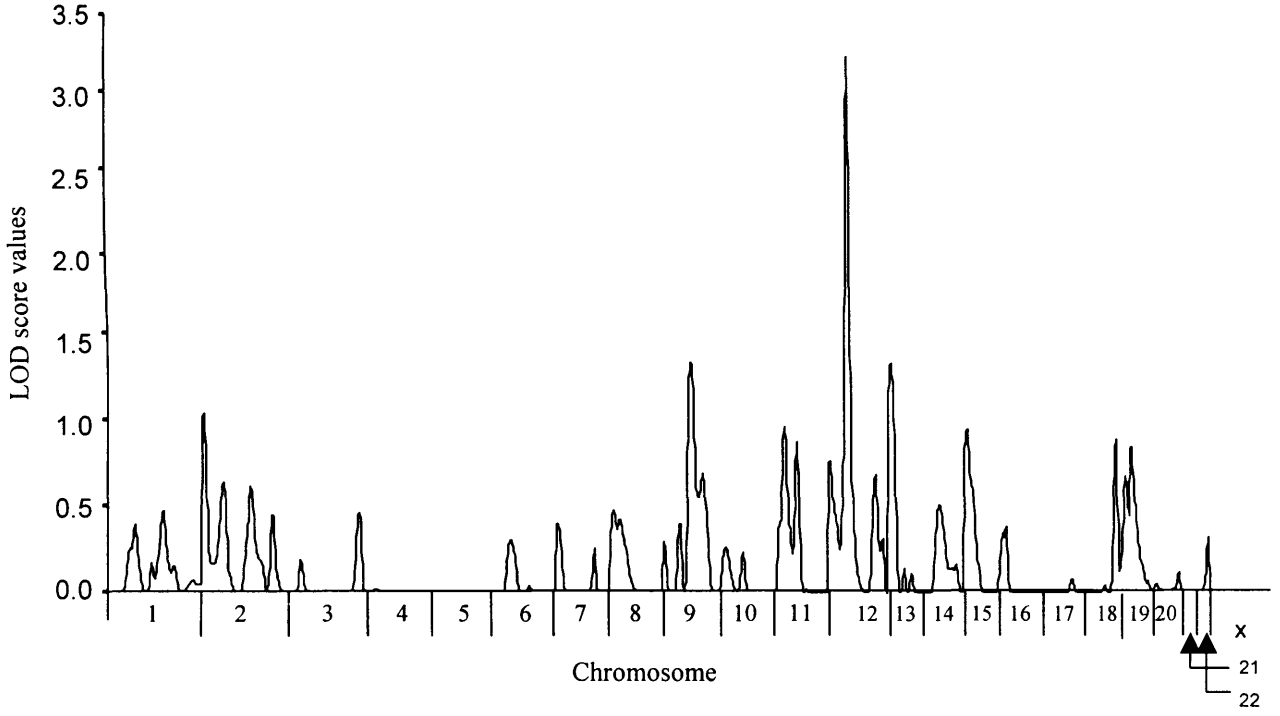


Figure 6.3: Initial Genome Scan. Scanning with 400 microsatellite markers at intervals of ~10cM demonstrated significant linkage to chromosome 12.

Four additional markers in the region were typed in order to confirm linkage and for fine mapping of the genetic interval. Marker D12S1698 gave a LOD score of 3.03 and multipoint analysis gave a maximum LOD score of 3.2 between markers D12S1640 and D12S1589 (Figure 6.4). Analysis using MERLIN gave similar results (LOD score of 3.07 p=0.00008 at marker D12S345 and 3.04 p=0.00009 at marker D12S1698). MERLIN was then used to generate 1500 simulated genome scans using the same conditions used during the analysis (families structure, phenotype, marker spacing, allele frequencies and missing data patterns). Sixty-seven simulations gave two consecutive markers with a LOD score > 3.0. This equates to a genome wide p value for the finding of 0.044. Heritability analysis (genetic variation/total variation) showed that

49 % of the total inter-individual variability of mean TRF length can be attributed to the locus on chromosome 12.

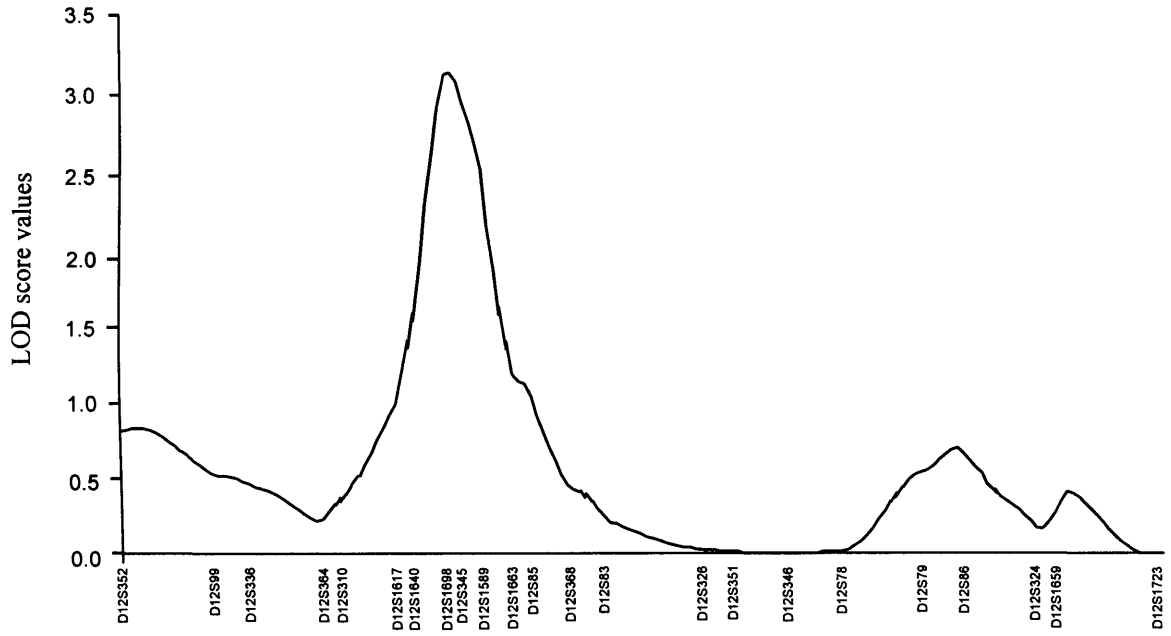


Figure 6.4: Additional Genome Scan. Four additional markers in the region were typed in order to confirm linkage and for fine mapping of the genetic interval. Marker D12S168 gave a LOD score of 3.03 for the marker D12S1698 and multipoint analysis gave a maximum LOD score of 3.2 between markers D12S1640 and D12S1589.

According to Ensembl the 1 LOD interval for the locus on chromosome 12 spans 13.2 Mb and contains 34 genes (42 counting isoforms and other predicted transcripts, see Table 6.3).

6.4 DISCUSSION

This study has demonstrated that telomere length is a highly heritable trait, and that a major locus on chromosome 12 determines almost half the inter-individual variation. Although the study cannot exclude the possibility that another major locus is responsible for the remainder of the heritable influence on mean TRF length, the more likely scenario is that this is due to the effect of several genes with small effects. The observed peaks with LOD scores between 1 and 1.5 on chromosomes 2, 9 and 13 (Figure 6.3), could harbour such loci, but additional studies would be necessary to confirm this.

Once again there was a wide degree of inter-individual variation in telomere length at all ages examined, in agreement with the results of the Chapters 3 and 4 (showing ranges of 3kb and 2kb respectively). An age-related decline in telomere length was also evident: the rates of attrition were slightly different between the sexes, with males losing approximately 30bp/year to just 17bp/year in females, although the difference did not reach significance. A difference in the age-adjusted mean TRF between males and females was also observed. Pre-menopausal women are known to be at a reduced risk of CVD (British Heart Foundation, 2003), and have longer telomeres than age-matched men (Benetos *et al.*, 2001; Cawthon *et al.*, 2003; Jeanclos *et al.*, 2000), partly due to the effect of oestrogen on telomerase. Telomerase is stimulated by oestrogen: there is an oestrogen response element on the catalytic subunit of the enzyme (Kyo *et al.*, 1999), and oestrogen receptors are present in vascular cells (Mendelsohn and Karas, 1999). However, the range of ages examined in this study is 47 – 82 years, thus many of the 92 women in the study may have been post-menopause. Therefore, the sex-related difference observed most likely reflects effects of oestrogen pre-menopause; when oestrogen is no longer stimulating telomerase, the rate of telomere attrition in women would have to exceed the rate in men in order to close the "gap" in length observed between the sexes. Figure 6.1 demonstrates that this was not the case: the rate of attrition was greater (although not significantly so) in men.

6.4.1 Inter-sibling correlation in mean TRF length

Telomere length has been shown to be a heritable trait in twin studies (Jeanclos *et al.*, 2000; Slagboom *et al.*, 1994). These studies examined 123 and 49 twin pairs respectively, but while both examined monozygotic (MZ) and dizygotic (DZ) twins,

only Slagboom *et al.* examined the correlation in the separate groups. This particular study demonstrated a higher correlation in MZ twins (0.78) than DZ twins (0.39), an unsurprising result, given that MZ twins are genetically identical: regardless of the precise *mode* of inheritance, they will have inherited telomeres of the same length. Any measured differences in telomere length between the twins at a later date likely reflect differing rates of attrition throughout life.

This study examined a total of 258 sib-pairs in order to estimate heritability at approximately 82%, a figure in close agreement with previous estimates of approximately 80% (Jeanclos *et al.*, 2000; Slagboom *et al.*, 1994). It is likely that this degree of heritability is due to a number of loci, each exerting an effect on telomere length. Linkage analysis using a genome wide scan provided the method for mapping individual loci.

6.4.2 Genome scan

The initial genome scan with 400 markers identified significant linkage to chromosome 12: a maximum LOD score of 3.2 was obtained using SOLAR, and 3.04 using MERLIN (a second software package was used in this study to validate the findings). MERLIN was then used to generate 1500 simulated genome scans using the same conditions used during the analysis (families structure, phenotype, marker spacing, allele frequencies and missing data patterns). Sixty-seven simulations gave two consecutive markers with a LOD score > 3.0, equating to a genome wide p value of 0.044 for our finding.

Heritability analysis has showed that almost half of the total inter-individual variability in mean telomere length can be attributed to this particular locus on chromosome 12, but what is controlling the other 50%? Inheritance of telomere length is a complex process that does not appear to follow simple Mendelian inheritance; rather, it is likely that there are a number of additional loci, each exerting an effect. This is reflected in the number of additional peaks, such as those on chromosomes 9 and 13. However, the fact that these both have LOD scores of just under 1.5 indicates that we cannot have the same degree of confidence that these loci are truly in linkage. Thus, the region identified on chromosome 12 harbours a major locus involved in the determination of telomere length.

Table 6.3: Genes within the one LOD drop region of the chromosome 12p11.2-q12 linkage peak.

Gene ID	*Description*
PPFIBP1	PTPRF Interacting Protein Binding Protein 1 Isoform 1
MRPS35	Mitochondrial Ribosomal Protein S35
YD40_HUMAN	YD40_Human
PTHLH	Parathyroid Hormone-Related Protein Precursor
NM_018318	Hypothetical Protein NM_018318
NM_018099	Hypothetical Protein NM_018099
NM_016570	Hypothetical protein similar to PTX1
NM_175861	Hypothetical protein ARG99 protein
IPO8	Importin 8
C1QDC1	C1Q Domain containing 1 Isoform L
DDX11	***DEAD/H Box Polypeptide 11; Yeast Chl1 Homolog***
Q9BZ57	Hypothetical protein Q9BZ57
NM_021238	Hypothetical protein similar to Tera Protein
NM_024799	Hypothetical protein NM_024799
NM_144973	Hypothetical protein NM_144973
AK3	Adenylate Kinase Isoenzyme 4
NM_173802	Hypothetical protein NM_173802
Q86X98	Hypothetical protein Q86X98
Q16776	***Gene Fragment For Histone H3***
NM 018169	Hypothetical protein NM 018169
BICD1	Bicaudal D Homolog 1
NM_139241	Actin-Filament Binding Protein Frabin.
DNM1L	Dynamin 1-Like Protein Isoform 3; Dynamin-Like Protein
NM_015936	Hypothetical protein NM_015936
PKP2	Plakophilin 2
NM 032834	Hypothetical protein NM 032834
NM 153634	Hypothetical protein similar to COPINE VIII
KIF21A	Ny-Ren-62 Antigen
ABCD2	Atp-Binding Cassette D2
NM 173599	Hypothetical protein NM 173599
SLC2A13	Proton Myo-Inositol Co-Transporter (Hmit).
Q8NCX9	Hypothetical protein Q8NCX9
CNTN1	Contactin Precursor
NM_013377	Hypothetical protein NM_013377

Candidate genes

Table 6.3 lists the genes within the one LOD drop region of the chromosome 12p11.2-q12 linkage peak. The strongest candidate is **DDX11**, a DNA helicase. DNA replication involves simultaneous synthesis of both the leading and lagging strands, resulting in shortening of the 5' end of the telomere. Replication is a complex process that requires the duplex DNA ahead of the DNA polymerase to be unwound by ATP-driven helicases. Many helicases have roles in maintaining telomeres and in telomere length control, as well as in segregation and DNA repair. Deficiencies in the RecQ helicases, BLM and RecQ4, result in Bloom and Rothmund-Thomson syndromes respectively: both syndromes are associated with increased occurrence of cancer (Mohaghegh and Hickson, 2001b). BLM and RecQ4 both interact with TRF2 (Opresko *et al.*, 2002; Stavropoulos *et al.*, 2002), indicating a role in telomere biology. Werner's syndrome (WS) displays early onset of many age-related pathologies, including type II diabetes and CVD (Martin, 1978). WS cells also display chromosomal rearrangements and deletions, and premature senescence. The syndrome is caused by loss of the RecQ helicase, WRN (Yu *et al.*, 1996). WS fibroblasts display accelerated telomere erosion (Schulz *et al.*, 1996) and WS lymphocytes show erratic telomere length distributions (Wyllie *et al.*, 2000). It has been suggested that WRN associates with telomeres when dissociation of the D-loop is required for replication and/or recombination (Opresko *et al.*, 2004). Interestingly, WRN unwinds a variety of DNA substrates, including the G-quadruplex (Mohaghegh *et al.*, 2001a), a structure that may occur at human telomeres *in vivo*.

A role for DDX11 in telomere length regulation is also supported by Zhu *et al.*, who demonstrated a 5cM region on mouse chromosome 2 that predominantly controls the observed species-specific telomere length regulation (Zhu *et al.*, 1998). Within this region a mouse homologue of the human novel helicase (NHL) gene (Bai *et al.*, 2000) was identified, and termed Regulator of Telomere Length (*Rtel*). Subsequent work suggests that *Rtel* is required to resolve higher order G-rich structures (such as G-quadruplexes), and lack of *Retl* results in variable loss of telomere repeats in undifferentiated embryonic stem (ES) cells, and maintenance of length at approximately 68% of that observed in the wild type ES cells (Ding *et al.*, 2004).

A second interesting candidate is **Q16776**, Gene Fragment for Histone H3. Histones are proteins present in the eukaryotic nucleus that help to wrap up the genetic material into

tightly packed chromosomes. Local chromatin structure can be reversibly changed from a condensed to a more accessible conformation by histone modification. Histone H3 contains certain amino acids, which are subject to modification, such as acetylation by histone acetyltransferases (Goll and Bestor, 2002). Histone deacetylases (HDACs) have the opposite effect, removing the acetyl group and promoting transcriptional repression. Histone modification may play a role in the telomere position effect, as Baur *et al.* (Baur *et al.*, 2001) have demonstrated that the suppression of certain genes located close to the telomere can be overcome by the action of trichostatin A, a histone deacetylase inhibitor. Thus, histones may function, in part, to regulate the precise conformation of the telomere, allowing/restricting access to the multitude of binding proteins and enzymes that exert an effect, and regulating the TPE.

6.4.3 Limitations of the study

The actual gene(s) exerting an effect on telomere length have yet to be identified. There are two strategies available for determining which genes(s) within the candidate region exert an effect on telomere length: single nucleotide polymorphism (SNP) genotyping and fine-mapping. Genes having a plausible role in telomere biology (such as DDX11 and Q16776) can be examined for SNPs. SNPs are naturally occurring variations affecting only a single nucleotide; they can be either synonymous (the mutation does not change the amino acid), or non- synonymous (where it does). They occur at a rate of approximately one SNP per kilobase of DNA, thus any one gene may contain several SNPs. By screening individuals from the two extremes of telomere length it may be possible to identify a SNP(s) that result in a functional variant. A large cohort can then be genotyped to examine any associations between particular SNPs and telomere length. The second approach is to carry out fine-mapping. This involves the use of larger numbers of micro-satellite markers to progressively narrow the candidate region, until it is of a suitable size for full sequence analysis. As in SNP genotyping, the chance of finding sequence differences may be increased by sequencing the region from individuals at both extremes of telomere length.

Interestingly, the findings of a locus on chromosome 12 do not support the recent proposal that the majority of inheritance of telomere length is X-linked (Nawrot *et al.*, 2004). This study on patterns of correlation within nuclear families (128 parents and 199 offspring) found that telomere length was similar between fathers and daughters,

mothers and sons, mothers and daughters, and among siblings, but not among fathers and sons. The authors note that the X chromosome harbours a number of genes that are important in telomere biology, such as the DKC1 gene encoding the protein dyskerin (important for stable accumulation of the hTR component of telomerase) (Mitchell *et al.*, 1999), and the gene encoding the angiotensin II receptor (AGTR2), the stimulation of which leads to enhance nitric oxide production (Volpe *et al.*, 2003). While the observations made by Nawrot *et al.* are interesting, their correlations in mean TRF length, particularly between father and both offspring, and mother and both offspring, are open to criticism. There appear to be a number of outliers across the study that could possibly skew the results. In this regard one would like to see a much larger study of the same type carried out to confirm the results, as the authors also acknowledged that they could not exclude the possibility that theirs was a chance finding.

Another feature of the present study that requires comment is the measurement of mean TRF length in sib pairs with CHD, a pragmatic choice as the initial genome scan data were already available in these subjects. As an association between shorter telomeres and risk of CHD has been shown in Chapters 3 and 5, and by Cawthon *et al.* (Cawthon *et al.*. 2003), the question arises as to whether this could have affected the findings. However, selection into the study from within the population of CHD patients is unlikely to be related either to telomere length or genotype. Furthermore, multipoint linkage analysis using CHD as a trait within the sample showed no evidence of significant or even suggestive linkage for any of the telomere length QTL peaks. Therefore, although confirmation in non-CHD patients is required, there is every reason to suppose that these findings would generalize.

In summary, a genome-wide scan of 173 families and 258 sib-pairs has demonstrated that telomere length is a highly heritable trait, and that a locus on chromosome 12 determines almost half the inter-individual variation in telomere length. Additional peaks on chromosomes 2, 9 and 13 may harbour loci accounting for the remainder of the variation. While there are several candidates within the region, more work is required to fine-map the region in order to more precisely identify the gene(s) involved.

GENERAL DISCUSSION

In the absence of telomerase, telomeres progressively shorten until a critical length is reached, at which point the cell enters senescence. Harley was the first to propose the telomere hypothesis of ageing (Harley *et al.*, 1992), suggesting that telomere length functions as the mitotic clock that regulates cellular lifespan. This has been supported by studies demonstrating progressive telomere shortening with age (Allsopp *et al.*, 1992; Chang and Harley, 1995; Vaziri *et al.*, 1993), and the observation that initial telomere length gives a strong indication of the replicative potential of the cell (Frenck *et al.*, 1998), thus, telomere length is regarded as a marker of "biological" ageing. Given that CHD is an age-related disorder, and telomere length has been shown to be shorter in subjects with triple vessel CAD compared to age and sex-matched controls (Samani *et al.*, 2001), it was hypothesised that CHD may be a disease of premature biological ageing.

7.1 Telomere length in WBCs, and the association with CHD

The observed differences in mean telomere length reported in Chapters 3 and 5 supports that observed by Samani *et al.* The estimated "biological" age difference between subjects with premature CHD and controls was in remarkable agreement between the three studies: 8.6 years (Samani *et al.*, 2001), 11.4 years (Chapter 3) and 9.4 years (Chapter 5). These results strongly suggest that CHD is a result of premature biological ageing, as subjects with CHD had a mean WBC telomere length that was similar to mean telomere length in healthy control subjects approximately 10 years older.

Inflammation is a fundamental process in atherosclerosis, thus shorter telomere length in WBCs may simply reflect increased cell turnover. However in Chapter 3, logistic regression analysis found that neither markers of inflammation, nor any other recognised risk factor for CHD, affected the association, suggesting that the shorter telomere length observed in WBCs is not simply an epiphenomenon. Furthermore, shorter telomeres have been demonstrated in coronary endothelial cells of patients with CHD than controls (Ogami *et al.*, 2004), and Cawthon *et al.* (Cawthon *et al.*, 2003) have demonstrated an association between shorter telomeres and an increase in mortality from CVD in a *prospective* study. These observations, combined with the significant association demonstrated in Chapter 5 between shorter mean telomere length at

baseline, and the subsequent development of CHD, suggest that short telomeres are a primary abnormality predisposing to CHD.

These associations demonstrated by Samani *et al.* (Samani *et al.*, 2001), and in Chapter 3, were made using TRF analysis of DNA obtained from circulating WBCs. TRF analysis provides a very crude analysis of telomere length, and reveals nothing of cell, or chromosome-specific, telomere length. In addition, the sub-telomeric region is captured by TRF analysis. The real-time PCR method used in Chapter 5 does not capture the sub-telomeric region, but is otherwise subject to similar criticism as it also estimates mean, rather than specific, telomere length. In addition, while these studies demonstrated strong associations, none have examined telomere length in other cell or tissue types.

The study of specific telomere length, and of other cell/tissues types, is important for a number of reasons. There is a high degree of inter- and intra-chromosomal, in addition to inter- and intra-cellular differences in telomere length, as illustrated by the "smear" obtained in TRF analysis, and the wide melt-curves obtained using real-time PCR. It has been suggested that senescence occurs when telomeres reach a critical length of 4-7kb (Itahana *et al.*, 2001), but it may be the presence of a ***single*** critically short telomere that induces senescence. Given that a chromosome-specific pattern of telomere length has been demonstrated (Graakjaer *et al.*, 2004), it would be interesting to examine individual telomeres in various cells from both patients with CHD, and healthy controls, to establish if there is a "profile" of chromosome-specific telomere length that is associated with CHD. Martens *et al.* (Martens *et al.*, 1998) have demonstrated that the telomere of chromosome 17p is consistently shorter than average: chromosome 17p contains both p53 and other potential tumour-suppressor genes, and is frequently lost in human cancers. Perhaps there is a specific telomere that is consistently shorter in subjects with CHD. Also, the telomere-position effect results in reversible silencing of genes located close to a telomere and although there is little evidence at present, the possibility remains that telomere shortening results in alterations in expression of genes implicated in atherogenesis.

There are PCR-based methods for the analysis of specific telomeres (Baird *et al.*, 2003) but these are often labour intensive, requiring specific primers to be designed for each telomere. An alternative approach is the use of quantitative fluorescence *in situ* hybridization (Q-FISH) with telomeric probes (Batliwalla *et al.*, 2000; Ferlicot *et al.*,

2003), a method that can be performed on fixed frozen tissue sections in order to assess telomere length. There are also a number of studies that could be carried out to demonstrate a correlation in length between different cell and tissue types. The simplest may be to estimate telomere length in DNA obtained from buccal swabs and WBCs, although this would reveal little about telomere length in the vasculature. A potentially more informative study along a similar line is currently being set up. Patients undergoing carotid bypass have a section of their internal mammary artery (IMA) removed, and used for the graft. Mean telomere length in DNA obtained from WBCs, healthy IMA, and atherosclerotic tissue taken from the carotid artery can be estimated, and correlations in length examined. While telomere length should be shortest in the atherosclerotis tissue, we would expect to find a correlation in length with the other tissues. Of course, if TRF or real-time PCR analysis were used they would still reveal nothing of individual telomere lengths.

7.2 What factors influence telomere length?

Telomere length at any given point in time will be a function of initial length and the rate of attrition (Figure 7.1). A number of studies have indicated that telomere length is a heritable trait (Jeanclos *et al.*, 2000; Slagboom *et al.*, 1994), and the correlations demonstrated between parents and offspring (R = 0.57, Chapter 4), and between sibs (R = 0.44, Chapter 6) strongly support this. These studies estimated heritability at approximately 80%. Interestingly, Chapter 4 demonstrated that the young, healthy offspring of subjects with premature CHD had a shorter mean telomere length in their WBCs than matched controls, perhaps indicating that inheritance of telomere length may also contribute to the familial aspect of CHD.

However, it may not be telomere length alone that accounts for this high estimate of heritability. A recent paper published by Schaetzlein *et al.* (Schaetzlein *et al.*, 2004) has raised an interesting possibility, regarding telomerase. This study demonstrated a burst of telomerase activity at the morula to blastocyst stage during embryogenesis, effectively resetting telomere length. This is consistent with the observed synchrony observed between foetal tissues at birth (Youngren *et al.*, 1998), although it would imply that from the blastocyst stage onward, all cells turnover at the same rate until birth. Assuming that the activity of telomerase is the same in all individuals, the length *after* the 'burst' will be a function of the length *before* it. However, if two individuals

possess variants of the telomerase gene with, for example, different rates of activity, the length set during this 'burst' may differ. A role for telomerase in the inheritance of telomere length may be supported by the results of Chapter 6, as a DNA helicase resides within the candidate region. DNA helicases unwind double-stranded DNA at the replication fork, thus defects impair the replication of DNA. It may be that inheritance of a variant of a helicase affects the activity of telomerase, both *in utero*, and throughout life. Alternatively, variations in the histone H3 (a second candidate in the region) may alter the chromatin structure such that the telomere complex is compromised, perhaps preventing telomerase from actually accessing the telomere at all.

While much work has focused on the inheritance of telomere length, and the action of telomerase, there are also a number of exogenous factors that influence the rate of attrition. If the rate of cell turnover increases there is, in the absence of telomerase, an increase in the rate of attrition as a direct result of replicative stress, perhaps the major contributor to telomere shortening. This is confirmed by studies demonstrating that increased shear wall stress results in accelerated telomere attrition in the abdominal aorta (Okuda *et al.*, 2003), and that there is as increase in endothelial cell turnover at sites of bifurcations (Kunz and Keim, 1975). A number of conventional risk factors for CHD also have a direct effect on telomere length. For example, damage caused by oxidative stress is repaired less well in telomeric DNA than elsewhere in the chromosome, and accelerates attrition, whereas antioxidants slow attrition (von Zglinicki T, 2002a) and homocysteine has been shown to increase the amount of telomeric DNA lost per population doubling (Xu *et al.*, 2000) (although it was shown to have no significant effect on mean telomere length in Chapter 3).

More work is clearly required to gain a better understanding of factors that influence telomere length. The candidate region identified on chromosome 12 requires further investigation, using either SNP genotyping or fine mapping, to identify the key gene(s) that may regulate telomere length. Further study of the additional regions identified (on chromosomes 2, 9 and 13) is also required, although the effects of these regions on regulation of length are likely to be considerably less than that of chromosome 12.

There are also other factors that may affect the functioning of a telomere. These include the numerous telomere-binding proteins that stabilise, and regulate the open and closing of the t-loop. The work by Minamino *at al* (Minamino *et al.*, 2002) demonstrated the effect of a TRF2-mutant on telomere biology, and this raises the possibility that

variations in other binding proteins may also affect telomere stability. These may be investigated using SNP analysis. The formation of the loop itself is dependent on the single-stranded, G-rich, 3'-overhang (Griffith *et al.*, 1999; Stansel *et al.*, 2001), thus differences in the length of the overhang may affect the ability to form *stable* loops. Differences in length could be estimated using either the oligonucleotide ligation assay (TOLA) (Cimino-Reale *et al.*, 2001; Cimino-Reale *et al.*, 2003), or primer-extension-nick-translation (PENT) (Makarov *et al.*, 1997), to investigate the possibility that the telomeres of those individuals with shorter overhangs are less capable of forming t-loops, thus exposing the telomere to the DNA-damage sensing pathways.

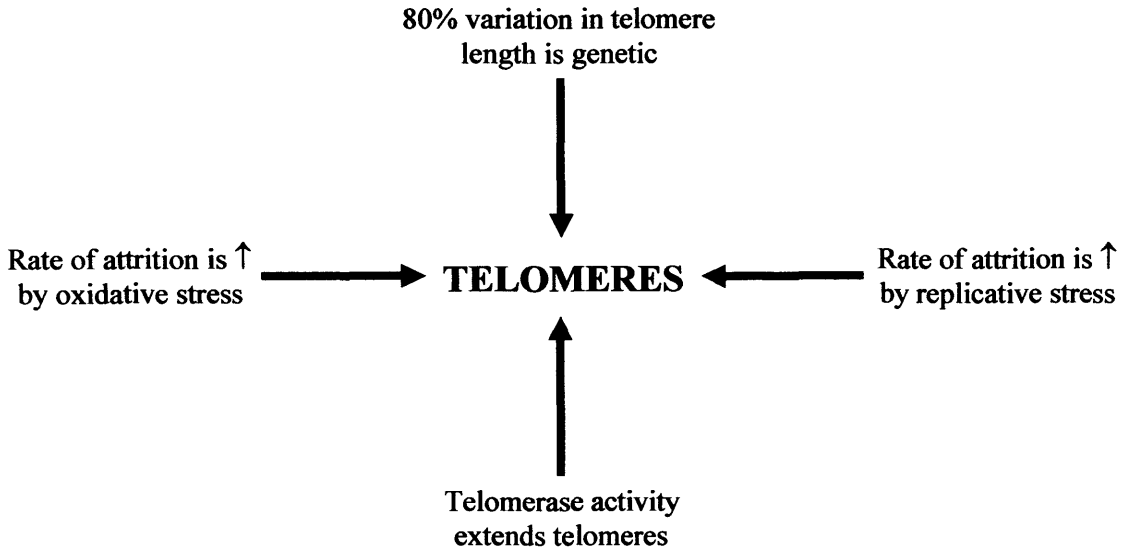


Figure 7.1: Summary of the factors influencing telomere length. Telomere length is a function of the inherited length and the effect of exogenous factors such as telomerase activity, replicative and oxidative stress.

7.3 How can shorter mean telomere length increase the risk of CHD?

The endothelial cell wall may provide the "missing link" between any given risk factor and its detrimental vascular effect (Bonetti *et al.*, 2003). A healthy vascular endothelium promotes and maintains vascular homeostasis by balancing the production of vasoconstrictors and vasodilators, and damage to the endothelium is thought to initiate the atherosclerotic process (Ross, 1999). Under normal conditions, there is little expression of pro-inflammatory markers, however, both the classic, and emerging risk

factors have been shown to initiate an inflammatory response through disruption of vascular homeostasis (Celermajer *et al.*, 1992; Fichtlscherer *et al.*, 2000; Prasad *et al.*, 2002; Sorensen *et al.*, 1994). Thus it is now believed that it is endothelial dysfunction as a result of damage caused by the presence of risk factors for CHD that is key in atherogenesis. If subjects exhibit short telomeres in their endothelial cells, they are prone to reach a critical length after fewer cell divisions, resulting in endothelial senescence. In the presence of risk factors for CHD that cause insult to the endothelium, this would occur even sooner, perhaps explaining why, in the presence of similar risk profiles, there is wide variety in the age-of-onset of CHD.

Burrig has demonstrated a link between endothelial cell senescence and atherosclerosis (Burrig KF, 1991), and Minamino *et al.* have recently demonstrated a direct link between telomere biology and endothelial dysfunction (Minamino *et al.*, 2002). These observations indicate that senescence is a feature of the atherosclerotic plaque, and that telomere shortening will result in endothelial dysfunction. The common pathway for unstable angina and MI is plaque destabilisation predisposing the plaque to rupture: destabilisation is the result of the interplay between inflammatory and pro-inflammatory mediators (Libby *et al.*, 2002). Factors such as NO may reduce endothelial expression of inflammatory mediators and adhesion molecules that increase the susceptibility to rupture (De Caterina *et al.*, 1995; Kubes *et al.*, 1991; Libby *et al.*, 2002), thus, a dysfunctional endothelium not only precedes plaque formation, but can also result in destabilisation of advanced plaques, leading to a clinical event. Damaged endothelium may be repaired by terminally-differentiated ECs adjoining the site of injury, although circulating EPCs can differentiate and migrate to the site to aid repair (Walter *et al.*, 2002). The overall effect is to accelerate the repair of the damaged endothelium, and restore vascular homeostasis. In this respect, the implication that statin treatment may accelerate endothelial repair through mobilisation of bone-marrow derived EPCs (Dimmeler *et al.*, 2001; Llevadot *et al.*, 2001; Vasa *et al.*, 2001a) is interesting, given that Chapter 5 demonstrated that treatment with pravastatin was associated with a significant reduction in clinical events only in those subjects at increased risk based on their telomere length.

It is likely to be the inheritance of shorter telomeres ***combined*** with the presence of certain cardiovascular risk factors that determines whether or not subjects will go on to develop atherosclerosis, and ultimately suffer a clinical event. This is supported to some

extent by a recent study showing that, in the presence of chronic hypertension, shorter white cell telomere length in WBCs was associated with increased predilection for carotid artery atherosclerosis (Benetos *et al.*, 2004). These data suggest that if two individuals are subjected to the same degree of hypertension, the individual who has inherited the shorter telomeres is much more likely to develop atheroma, due to premature endothelial senescence. Shorter telomeres alone may be insufficient to result in premature MI, but in concert with hypertension, or enhanced platelet reactivity, may result in the early plaque formation that can ultimately lead to an occlusive event. Thus, the correlation in telomere length between parents and offspring in Chapter 4 may be less to do with the inheritance of length *per se*, and more to do variants of the hTERT gene, or other as yet unidentified factors (such as DDX11), that regulate telomere length, in conjunction with the presence of risk factors for CHD.

Telomere shortening may also help to explain the "Barker" hypothesis. The theory suggests that the "catch-up" growth that occurs during early childhood, as a result of pre-term birth and low birth-weight, somehow "programmes" the development of various risk factors that are key determinants of CHD (Barker, 1989). Clearly, if two individuals are born pre-term, and experience the same degree of accelerated growth to "catch-up" in the first year of life, both will have lost additional telomeric DNA, but the individual that inherited shorter telomeres will be even closer to the length at which senescence occurs. This may account for the increased risk of CHD observed in individuals who are born small for gestational age.

7.4 Could telomere length be a therapeutic target?

Telomere length is dynamic, representing a balance between those factors that reduce length with the telomerase activity that has the potential to extend it. In most somatic cells the balance is tipped in the favour of telomere attrition, as they do not express the catalytic sub-unit of telomerase. Even in those cells that do, the level of activity is insufficient to counteract the rate of attrition, thus telomerase activity simply slows the rate, delaying the onset of senescence. If shortening of the telomeres in ECs to a critical length results in senescence, with the resulting atherogenic changes, any factor that can delay the onset of senescence will postpone those changes. Thus, modification of telomere length may provide a novel therapeutic target, particularly in the ECs, but also in other cells of the vasculature. For example, Bentos *et al.* (Benetos *et al.*, 2001) have

shown that men with shorter telomeres in their WBCs are more likely to exhibit high PP and PWV, markers of arterial stiffness that independently predicts cardiovascular risk (Blacher *et al.*, 1999; Laurent *et al.*, 2001). If WBC telomere length does indeed correlate with length in other tissues, telomere attrition resulting in senescence may also contribute to arterial stiffness. By manipulation of telomere length, the onset of the age-related changes that occur in the vasculature may be delayed, and this would be particularly important for those endowed with shorter than average telomeres.

Two key observations in this thesis suggest that telomere length may provide a valuable therapeutic target. The finding that statin treatment reduced the number of clinical events, only in those at increased risk based on their telomere length, is particularly interesting, as the primary mode of action for statins is inhibition of cholesterol synthesis. However, it has been proposed that statins are capable of increasing the number of circulating EPCs, resulting in accelerated endothelial repair (Walter *et al.*, 2002). Further work is required to elucidate the precise mechanism by which statins reduce clinical events in those with short telomeres, and to investigate whether other, similar treatments also influence endothelial function.

The second observation suggesting that telomere length may be a suitable target for therapy is the finding of a candidate region responsible for almost 50% of the inter-individual differences in telomere length on chromosome 12. Once the gene(s) responsible is identified, it will be necessary to investigate the mechanisms by which it exerts its effect. The potential roles of the two most promising candidates have already been discussed: helicases unwind dsDNA at the replication fork, and histones affect chromatin conformation. Both have potential roles in regulating telomere biology, thus may also provide substrates for therapy.

However, while the manipulation of telomere length to delay the onset of cellular senescence may provide potential therapeutic benefits, there are also potential adverse effects. The balance between telomere extension and attrition may favour attrition for a vital reason. Telomerase is activated in a number of cancers (Hiyama *et al.*, 1996; Shay and Bacchetti, 1997) and evidence suggests that replicative senescence functions as a tumour-suppressor mechanism. (Newbold *et al.*, 1982) (Sager, 1991; Shay *et al.*, 1991). Campisi (Campis, 1997) has summarised the resulting problem: if replicative senescence acts as a tumour-suppression mechanism, how can we reverse the deleterious effects (compromised tissue function) without reversing the beneficial

effects (tumour suppression)? If the telomere is ever to be considered as a therapeutic target, a great deal of work will be required to understand how to modulate length and stability, without tipping the balance in favour of tumour formation.

7.5 The "Telomere" Hypothesis of CHD

Taken together, the findings presented support a novel "telomere" hypothesis of CHD, indicating that telomere biology is intimately linked to the genetic aetiology and pathogenesis of CHD. Specifically, the findings suggest that:

1. Those individuals inheriting shorter telomeres will experience the onset of senescence, in any cell type, earlier than in those individuals inheriting longer telomeres, due to decreased replicative capacity. The presence of various cardiovascular risk factors places replicative stress on the endothelium in particular. Once the ability to repair has been exhausted, endothelial senescence, along with atherogenic changes to vascular homeostasis, will occur. Thus, those individuals born with shorter telomeres may be at increased risk of CHD.
2. Rather than inheriting candidate genes that predispose to CHD, perhaps a more global, structural property of the genetic material that is inherited may explain the familial basis of CHD.
3. Given that telomere length predicts replicative capacity, variation in telomere length may provide, at least in part, an explanation for the variable age of onset of CHD.

Importantly, the findings provide several new avenues for future research.

REFERENCES

Abecasis, G.R., Cherny, S.S., Cookson, W.O., and Cardon, L.R. GRR: graphical representation of relationship errors. *Bioinformatics* 2000; **17**: 742-743.

Abecasis, G.R., Cherny, S.S., Cookson, W.O., and Cardon, L.R. Merlin - rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002; **30**: 97-101.

Adlard, P.A., Perreau, V.M., Engesser-Cesar, C., and Cotman, C.W. The timecourse of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. *Neurosci.Lett.* 2004; **363**: 43-48.

Aikata, H., Takaishi, H., Kawakami, Y., Takahashi, S., Kitamoto, M., Nakanishi, T., Nakamura, Y., Shimamoto, F., Kajiyama, G., and Ide, T. Telomere reduction in human liver tissues with age and chronic inflammation. *Experimental Cell Research.* 2000; **256**: 578-582.

Airaksinen, K.E., Salmela, P.I., Linnaluoto, M.K., Ikaheimo, M.J., Ahola, K., and Ryhanen, L.J. Diminished arterial elasticity in diabetes: association with fluorescent advanced glycosylation end products in collagen. *Cardiovasc.Res* 1993; **27**: 942-945.

Akkad, A.A., Halligan, A.W., Abrams, K., and al-Azzawi, F. Differing responses in blood pressure over 24 hours in normotensive women receiving oral or transdermal estrogen replacement therapy. *Obstet.Gynecol.* 1997; **89**: 97-103.

Al-Abed, Y., Mitsuhashi, T., Li, H. Inhibition of advanced glycation end-product formation by acetaldehyde: role in the cardioprotective effect of ethanol. *Proc.Natl.Acad.Sci.USA.* 1999; **96**: 2385-2390.

Allsopp, R.C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E.V., Futcher, A.B., Grieder, C.W., and Harley, C.B. Telomere length predicts replicative capacity of human fibroblasts. *Proc.Natl.Acad.Sci.USA.* 1992; **89**: 10114-10118.

Almasy, L., and Blangero, J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am.J Hum.Genet* 1998; **62**: 1198-1211.

Anderson, T.J. Assessment and treatment of endothelial dysfunction in humans. *J Am.Coll.Cardiol.* 1999; **34**: 631-638.

Anderson, J.L., and Muhlestein, J.B. Antibiotic trials for coronary heart disease. *Tex.Heart Inst.J.* 2004; **31**: 33-38.

Angles-Cano, E., de la Pena, D.A., and Loyau, S. Inhibition of fibrinolysis by lipoprotein(a). *Ann N Y Acad Sci* 2001; **936**: 261-275.

Artlett, C.M., Black, C.M., Briggs, D.C., Stevens, C.O., and Welsh, K.I. Telomere reduction in scleroderma patients: a possible cause for chromosomal instability. *British Journal of Rheumatology.* 1996; **35**: 732-737.

Austin, M.A., Rodriguez, B.L., McKnight, B., McNeely, M.J., Edwards, K.L., Curb, J.D., and Sharp, D.S. Low-density lipoprotein particle size, triglycerides, and high-density lipoprotein cholesterol as risk factors for coronary heart disease in older Japanese-American men. *American Journal of Cardiology.* 2000; **86**: 412-416.

Aviv, A. Hypothesis: pulse pressure and human longevity. *Hypertension* 2001; 37: 1060-1066.

Bai, C., Connolly, B., Metzker, M.L., Hilliard, C.A., Liu, X., Sandig, V., Soderman, A., Galloway, S.M., Liu, Q., Austin, C.P., and Caskey, C.T. Overexpression of M68/DcR3 in human gastrointestinal tract tumors independent of gene amplification and its location in a four-gene cluster. *Proc.Natl.Acad.Sci.U.S.A* 2000; **97**: 1230-1235.

Bailey, S.M., Meyne, J., Chen, D.J., Kurimasa, A., Li, G.C., Lehnert, B.E., and Goodwin, E.H. DNA double-strand break repair proteins are required to cap the ends of mammalian chromosomes. *Proc.Natl.Acad Sci U.S.A* 1999; **96**: 14899-14904.

Baird, D.M., Rowson, J., Wynford-Thomas, D., and Kipling, D. Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat Genet* 2003a; **33**: 203-207.

Barath, P., Fishbein, M.C., Cao, J., Berenson, J., Helfant, R.H., and Forrester, J.S. Detection and localization of tumor necrosis factor in human atheroma. *Am.J Cardiol.* 1990; **65**: 297-302.

Barker, D.J.P. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; **2**: 577-580.

Barker, D.J.P., Meade, T.W., Fall, C.H., Lee, A., Osmond, C., Phipps, K., and Stirling, Y. Relation of fetal and infant growth to plasma fibrinogen and factor VII concentrations in adult life. *BMJ* 1992; **304**: 148-152.

Barker, D.J.P., Martyn, C.N., Osmond, C., Hales, C.N., and Fall, C.H. Growth in utero and serum cholesterol concentrations in adult life. *BMJ* 1993; **307**: 1524-1527.

Barker, D.J.P., Forsen, T., Uutela, A., Osmond, C., Eriksson, J.G. Size at birth and resilience to the effects of poor living conditions in adult life: a longitudinal study. *Br.Med.J* 2001; **323**: 1273-1276.

Barker, D.J.P., Eriksson, J.G., Forsen, T., and Osmond, C. Fetal origins of adult disease: strength of effects and biological basis. *Int.J Epidemiol.* 2002; **31**: 1235-1239.

Barker, D.J.P. Forsen, T., Eriksson, J.G., Osmond, C. Growth and living conditions in childhood and hypertension in adult life: a longitudinal study. *J.Hypertens.* 2002; 20: 1951-1956.

Batliwalla, F.M., Rufer, N., Lansdorp, P.M., and Gregersen, P.K. Oligoclonal expansions in the CD8(+)CD28(-) T cells largely explain the shorter telomeres detected in this subset: analysis by flow fish. *Human Immunology* 2000; **61**: 951-8.

Baumann, P., and Cech, T.R. Pot1, the putative telomere end-binding protein in fission yeast and humans. *Science* 2001; **292**: 1171-1175.

Baur, J.A., Zou, Y., Shay, J.W., and Wright, W.E. Telomere position effect in human cells. *Science* 2001; **292**: 2075-2077.

Baynes, J.W., and Thorpe, S.R. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 1999; **48**: 1-9.

Beljic, T., Babic, D., Marinkovic, J., and Prelevic, G.M. The effect of hormone replacement therapy on diastolic left ventricular function in hypertensive and normotensive postmenopausal women. *Maturitas* 1998; **29**: 229-238.

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., Labat, C., Bean, K., and Aviv, A. Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension* 2001; **37**: 381-5.

Benetos, A., Gardner, J.P., Zureik, M., Labat, C., Xiaobin, L., Adamopoulos, C., Temmar, M., Bean, K.E., Thomas, F., and Aviv, A. Short telomeres are associated with increased carotid atherosclerosis in hypertensive subjects. *Hypertension* 2004; **43**: 182-185.

Bennett, M.R., Evan, G.I., and Schwartz, S.M. Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin.Invest* 1995; **95**: 2266-2274.

Benowitz, N.L. The role of nicotine in smoking-related cardiovascular disease. *Preventive Medicine.* 1997; **26**: 412-417.

Berlin, J.A., and Colditz, G.A. A meta-analysis of physical activity in the prevention of coronary heart disease. *Am.J Epidemiol.* 1990; **132**: 612-628.

Bestilny, L.J., Gill, M., John, M., Christopher, H., and Riabowol, K.T. Accelerated replicative senescence of the peripheral immune system induced by HIV infection. *AIDS* 2000; **14**: 771-780.

Beyzade, S., Zhang, S., Wong, Y.K., Day, I.N., Eriksson, P., and Ye, S. Influences of matrix metalloproteinase-3 gene variation on extent of coronary atherosclerosis and risk of myocardial infarction. *Journal of the American College of Cardiology.* 2003; **41** : 2130-2137.

Bharadwaj, D., Stein, M. P., Volzer, M., Mold, C., and Du Clos, T.W. The major receptor for C-reactive protein on leukocytes is fcgamma receptor II. *J Exp.Med* 1999; **190**: 585-590.

Bianchi, A., and de Lange, T. Ku binds telomeric DNA in vitro. *J Biol Chem.* 1999; **274**: 21223-21227.

Bierman, E.L. The effect of donor age on the in vitro life span of cultured human arterial smooth-muscle cells. *In Vitro* 1978; **14**: 951-955.

Bjorntorp, P. Metabolic implications of body fat distribution. *Diabetes Care* 1991; **14**: 1132-1143.

Blacher, J., Guerin, A.P., Pannier, B., Marchais, S.J., Safar, M.E., and London, G. M. Impact of aortic stiffness on survival in end-stage renal disease. *Circulation* 1999; **99** : 2434-2439.

Bobik, A., Agrotis, A., Kanellakis, P., Dilley, R., Krushinsky, A., Smirnov, V., Tararak, E., Condron, M., and Kostolias, G. Distinct patterns of transforming growth factor-beta isoform and receptor expression in human atherosclerotic lesions. Colocalization implicates TGF-beta in fibrofatty lesion development. *Circulation* 1999; **99**: 2883-2891.

Boekholdt, S.M., Trip, M.D., Peters, R.J., Engelen, M., Boer, J.M., Feskens, E.J., Zwinderman, A. H., Kastelein, J.J., and Reitsma, P.H. Thrombospondin-2 polymorphism is associated with a reduced risk of premature myocardial infarction. *Arteriosclerosis, Thrombosis & Vascular Biology.* 2002; **22**: e24-e27.

Bonetti, P.O., Lerman, L.O., and Lerman, A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* 2003; **23**: 168-175.

Boring, L., Gosling, J., Cleary, M., and Charo, I. F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature* 1998; **394**: 894-897.

Bots, M.L., Hoes, A.W., Koudstaal, P.J., Hofman, A., and Grobbee, D.E. Common carotid intima-media thickness and risk of stroke and myocardial infarction: the Rotterdam Study. *Circulation* 1997; **96**: 1432-1437.

Boulay, J.L., Reuter, J., Ritschard, R., Terracciano, L., Herrmann, R., and Rochlitz, C. Gene dosage by quantitative real-time PCR. *Biotechniques* 1999; **27**: 228-30, 232.

Boushey, C.J., Beresford, S.A., Omenn, G.S., and Motulsky, A.G. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* 1995; **274**: 1049-1057.

Brand, K., Banka, C.L., Mackman, N., Terkeltaub, R.A., Fan, S.T., and Curtiss, L.K. Oxidized LDL enhances lipopolysaccharide-induced tissue factor expression in human adherent monocytes. *Arterioscler Thromb* 1994; **14**: 790-797.

Brattström, L. Common mutation in the methylenetetrahydrofolate reductase gene offers no support for mild hyperhomocysteinemia being a causal risk factor for cardiovascular disease. *Circulation* 1997; **96**: 3805-3807.

Brattström, L., Wilcken, D.E.L., Ohrvik, J. and Brudin, L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinaemia but not to vascular disease: the results of a meta-analysis. Circulation 1998; **98:** 2520–2526.

Brien, T.P., Kallakury, B.V., Lowry, C.V., Ambros, R.A., Muraca, P.J., Malfetano, J.H., and Ross, J.S. Telomerase activity in benign endometrium and endometrial carcinoma. *Cancer Res* 1997; **57**: 2760-2764.

British Heart Foundation. Coronary heart disease statistics - British Heart Foundation Statistics Database 2003.

Broccoli, D., Young, J.W., and de Lange, T. Telomerase activity in normal and malignant hematopoietic cells. *Proc.Natl.Acad Sci U.S.A* 1995; **92**: 9082-9086.

Brody, T.M., Larner, J., Minneman, K.P., and Neu, H.C. Human Pharmacology - Molecular to Clinical. 1994; **2**:

Brosnihan, K.B., Weddle, D., Anthony, M.S., Heise, C., Li, P., and Ferrario, C.M. Effects of chronic hormone replacement on the renin-angiotensin system in cynomolgus monkeys. *J Hypertens.* 1997; **15**: 719-726.

Brown, L.A., Nunez, D.J., and Wilkins, M.R. Differential regulation of natriuretic peptide receptor messenger RNAs during the development of cardiac hypertrophy in the rat. *J Clin.Invest* 1993; **92**: 2702-2712.

Brown, W.R., MacKinnon, P.J., Villasante, A., Spurr, N., Buckle, V.J., and Dobson, M.J. Structure and polymorphism of human telomere-associated DNA. *Cell* 1990; **63**: 119-132.

Bruschke, A.V., Kramer, J.R., Jr., Bal, E T., Haque, I.U., Detrano, R.C., and Goormastic, M. The dynamics of progression of coronary atherosclerosis studied in 168 medically treated patients who underwent coronary arteriography three times. *Am.Heart J* 1989; **117**: 296-305.

Bryan, T.M., Englezou, A., Dalla-Pozza, L., Dunham, M.A., and Reddel, R.R. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 1997; **3**: 1271-1274.

Bryan, T.M., Englezou, A., Dunham, M.A., and Reddel, R.R. Telomere length dynamics in telomerase-positive immortal human cell populations. *Experimental Cell Research* 1998; **239**: 370-378.

Burke, A.P., Farb, A., Malcom, G.T., Liang, Y., Smialek, J.E., and Virmani, R. Plaque rupture and sudden death related to exertion in men with coronary artery disease. *JAMA* 1999; **281**: 921-926.

Burns, J., Crozier, A., and Lean, M.E. Alcohol consumption and mortality: is wine different from other alcoholic beverages?. [Review] [49 refs]. *Nutrition Metabolism & Cardiovascular Diseases.* 2001; **11**: 249-258.

Burr, D., Doss, H., Cooke, G.E., and Goldschmidt-Clermont, P.J. A meta-analysis of studies on the association of the platelet PlA polymorphism of glycoprotein IIIa and risk of coronary heart disease. *Statistics in Medicine.* 2003; **22**: 1741-1760.

Burrig, K.F. The endothelium of advanced arteriosclerotic plaques in humans. *Arterioscler Thromb* 1991; **11**: 1678-1689.

Cai, H. and Harrison, D.G. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ.Res* 2000; **87**: 840-844.

Callen, E., Samper, E., Ramirez, M.J., Creus, A., Marcos, R., Ortega, J.J., Olive, T., Badell, I., Blasco, M.A., and Surralles, J. Breaks at telomeres and TRF2-independent end fusions in Fanconi anemia. *Human Molecular Genetics* 2002; **11**: 439-44.

Calori, G., D'Angelo, A., Della, Valle P., Ruotolo, G., Ferini-Strambi, L., Giusti, C., Errera, A., and Gallus, G. The effect of cigarette-smoking on cardiovascular risk factors: a study of monozygotic twins discordant for smoking. *Thrombosis & Haemostasis.* 1996; **75**: 14-18.

Campis, J. Aging and cancer: the double-edged sword of replicative senescence. *J.Am.Geriatr.Soc.* 1997; 45: 482-488.

Campis, J. and Dimri G.P. Control of replicative senescence. In: Schneider EL, Rowe J, eds. Handbook of the Biology of Aging. New York: Academic Press; 1996:121-149.

Campos, H., Genest, J.J., Jr., Blijlevens, E., McNamara, J.R., Jenner, J.L., Ordovas, J.M., Wilson, P.W., and Schaefer, E.J. Low density lipoprotein particle size and coronary artery disease. *Arteriosclerosis & Thrombosis.* 1992; **12**: 187-195.

Carr, S. C., Farb, A., Pearce, W. H., Virmani, R., and Yao, J. S. Activated inflammatory cells are associated with plaque rupture in carotid artery stenosis. *Surgery* 1997; **122**: 757-763.

Carrel, A. and Ebeling, A.H. Age and multiplication of fibroblasts. *J.Exp.Med.* 1921; **34**: 599-606.

Casscells, W., Naghavi, M., and Willerson, J.T. Vulnerable atherosclerotic plaque: a multifocal disease. *Circulation* 2003b; **107**: 2072-2075.

Cawthon, R.M. Telomere measurement by quantitative PCR. *Nucleic Acids Research* 2002; 30: ε47-

Cawthon, R.M., Smith, K.R., O'Brien, E., Sivachenko, A., and Kerber, RA Association between telomere length in blood and mortality in people aged 60 years or older. *The Lancet* 2003; **361**:

Celermajer, D.S., Sorensen, K.E., Gooch, V.M., Spiegelhalter, D.J., Miller, O.I., Sullivan, I.D., Lloyd, J.K., and Deanfield, J.E. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992; **340**: 1111-1115.

Celermajer, D.S., Sorensen, K.E., Bull, C., Robinson, J., and Deanfield, J.E. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am.Coll.Cardiol.* 1994a; **24**: 1468-1474.

Celermajer, D.S., Sorensen, K.E., Spiegelhalter, D.J., Georgakopoulos, D., Robinson, J., and Deanfield, J.E. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am.Coll.Cardiol.* 1994b; **24**: 471-476.

Cenarro, A., Artieda, M., Castillo, S., Mozas, P., Reyes, G., Tejedor, D., Alonso, R., Mata, P., Pocovi, M., Civeira, F., and Spanish FH group. A common variant in the ABCA1 gene is associated with a lower risk for premature coronary heart disease in familial hypercholesterolaemia. *Journal of Medical Genetics.* 2003; **40**: 163-168.

Cercato, C., Mancini, M.C., Arguello, A.M., Passos, V.Q., Villares, S.M., and Halpern, A. Systemic hypertension, diabetes mellitus, and dyslipidemia in relation to body mass index: evaluation of a Brazilian population. *Rev.Hosp.Clin.Fac.Med.Sao Paulo* 2004; **59**: 113-118.

Cermak, J., Key, N.S., Bach, R.R., Balla, J., Jacob, H.S., and Vercellotti, G.M. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. *Blood* 1993; **82**: 513-520.

Chait, A, Iverius, P, and Brunzell, JD *J.Clin.Invest.* 1982; **69**: 490-493.

Chakraverty, R.K. and Hickson, I.D. Defending genome integrity during DNA replication: a proposed role for RecQ family helicases. *Bioessays* 1999; **21**: 286-294.

Chan, S.W., and Blackburn, E.H. New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene* 2002; **21**: 553-563.

Chang, E., and Harley, C.B. Telomere length and replicative aging in human vascular tissues. *Proc.Natl.Acad.Sci.USA* 1995; **92**: 11190-11194.

Chang, Z.F. and Chen, K.Y. Regulation of ornithine decarboxylase and other cell cycle-dependent genes during senescence of IMR-90 human diploid fibroblasts. *J Biol Chem.* 1988; **263**: 11431-11435.

Chappey, O, Dosquet, C, and Wautier, JL Advanced glycation end products, oxidative stress and vascular lesions. *Eur.J.Clin.Invest.* 1997; **27**: 97-108.

Chen, Y.H., Chen, Y.L., Lin, S.J., Chou, C.Y., Mar, G.Y., Chang, M.S., and Wang, S.P. Electron microscopic studies of phenotypic modulation of smooth muscle cells in coronary arteries of patients with unstable angina pectoris and postangioplasty restenosis. *Circulation* 1997; **95**: 1169-1175.

Cherif, H., Tarry, J.L., Ozanne, S.E., and Hales, C.N. Ageing and telomeres: a study into organ- and gender-specific telomere shortening. *Nucleic Acids Res* 2003; **31**: 1576-1583.

Chi, N.W., and Lodish, H.F. Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles. *Journal of Biological Chemistry* 2000; **275**: 38437-44.

Christensen, K., Vaupel, J.W., Holm, N.V., and Yashin, A.I. Mortality among twins after age 6: fetal origins hypothesis versus twin method. *BMJ* 1995; **310**: 432-436.

Cimino-Reale, G., Pascale, E., Alvino, E., Starace, G., and D'Ambrosio, E. Long telomeric C-rich 5'-tails in human replicating cells. *J Biol Chem.* 2003; **278**: 2136-2140.

Cimino-Reale, G., Pascale, E., Battiloro, E., Starace, G., Verna, R., and D'Ambrosio, E. The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res* 2001; **29**: E35-

Ciruzzi, M., Schargrodsky, H., Rozlosnik, J., Pramparo, P., Delmonte, H., Rudich, V., Piskorz, D., Negri, E., Soifer, S., and La Vecchia, C. Frequency of family history of acute

myocardial infarction in patients with acute myocardial infarction. Argentine FRICAS (Factores de Riesgo Coronario en America del Sur) Investigators. *Am.J Cardiol.* 1997; **80**: 122-127.

Colditz, G.A., Rimm, E.B., Giovannucci, E., Stampfer, M J., Rosner, B., and Willett, W.C. A prospective study of parental history of myocardial infarction and coronary artery disease in men. *Am.J Cardiol.* 1991; **67**: 933-938.

Colgin, L.M., Baran, K., Baumann, P., Cech, T.R., and Reddel, R.R. Human POT1 Facilitates Telomere Elongation by Telomerase. *Curr.Biol* 2003; **13**: 942-946.

Comi, P., Chiaramonte, R., and Maier, J.A. Senescence-dependent regulation of type 1 plasminogen activator inhibitor in human vascular endothelial cells. *Exp.Cell Res.* 1995; **219** : 304-308.

Corash, L., Tan, H., and Gralnick, H.R. Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure. *Blood* 1977; **49**: 71-87.

Cybulsky, M.I., Iiyama, K., Li, H., Zhu, S., Chen, M., Iiyama, M., Davis, V., Gutierrez-Ramos, J. C., Connelly, P. W., and Milstone, D. S. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. *J Clin.Invest* 2001; **107**: 1255-1262.

Davies, M.J., Woolf, N., Rowles, P.M., and Pepper, J. Morphology of the endothelium over atherosclerotic plaques in human coronary arteries. Br Heart J 1988; 60: 459-464.

Davignon, J., Grogg, R.E., Sing, C.F. Apolipoprotein E polymorphisms and atherosclerosis. Arteriosclerosis. 1998; 8:1-21.

De Backer, J., Mak, R., De Bacquer, D., Van Renterghem, L., Verbraekel, E., Kornitzer, M., and De Backer, G. Parameters of inflammation and infection in a community based case-control study of coronary heart disease. *Atherosclerosis* 2002; **160**: 457-463.

De Bono, D.P. Olovnikov's clock: telomeres and vascular biology. *Heart* 1998; **80**: 110-11.

De Caterina, R., Libby, P., Peng, H.B., Thannickal, V.J., Rajavashisth, T.B., Gimbrone, M.A., Jr., Shin, W.S., and Liao, J.K. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin.Invest* 1995; **96**: 60-68.

de Lange, T., Shiue, L., Myers, R.M., Cox, D.R., Naylor, S.L., Killery, A.M., and Varmus, H.E. Structure and variability of human chromosome ends. *Mol.Cell Biol* 1990; **10**: 518-527.

de Lange, T. Cell biology. Telomere capping--one strand fits all. *Science* 2001; **292**: 1075-1076.

de Lange, T. Protection of mammalian telomeres. *Oncogene* 2002; **21**: 532-540.

Delanghe, J.R., Langlois, M.R., De Bacquer, D., Mak, R., Capel, P., Van Renterghem, L., and De Backer, G. Discriminative value of serum amyloid A and other acute-phase proteins for coronary heart disease. *Atherosclerosis* 2002; **160**: 471-476.

Department of Health National Service Framework for Coronary Heart Disease. 2000;

Dietschy, J.M. Theoretical considerations of what regulates low-density lipoprotein and high-density lipoprotein cholesterol. *American Journal of Clinical Nutrition* 1997; **65**: 1581-1589.

Dilman, V.M. The Law of Deviation of Homeostasis and Diseases of Aging. 1981;

Dilman, V.M., Revskoy, S.Y., and Golubev, A.G. Neuroendocrine-ontogenetic mechanism of aging: toward an integrated theory of aging. *Int.Rev Neurobiol.* 1986; **28**: 89-156.

Dimmeler, S., Aicher, A., Vasa, M., Mildner-Rihm, C., Adler, K., Tiemann, M., Rutten, H., Fichtlscherer, S., Martin, H., and Zeiher, A. M. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin.Invest* 2001; **108**: 391-397.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., and Pereira-Smith, O. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* 1995; **92**: 9363-9367.

Ding, H., Schertzer, M., Wu, X., Gertsenstein, M., Selig, S., Kammori, M., Pourvali, R., Poon, S., Vulto, I., Chavez, E., Tam, P.P., Nagy, A., and Lansdorp, P.M. Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. *Cell* 2004; **117**: 873-886.

Djousse, L., Myers, R.H., Coon, H., Arnett, D.K., Province, M.A., and Ellison, R.C. Smoking influences the association between apolipoprotein E and lipids: the National Heart, Lung, and Blood Institute Family Heart Study. *Lipids.* 2000; **35**: 827-831.

Drake, T.A., Morrissey, J.H., and Edgington, T.S. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am.J Pathol* 1989; **134**: 1087-1097.

Drake, T.A., Hannani, K., Fei, H.H., Lavi, S., and Berliner, J.A. Minimally oxidized low-density lipoprotein induces tissue factor expression in cultured human endothelial cells. *Am.J Pathol* 1991; **138**: 601-607.

Duan, J., Martinez, M., Sanders, A.R., Hou, C., Saitou, N., Kitano, T., Mowry, B.J., Crowe, R.., Silverman, J.M., Levinson, D.F., and Gejman, P.V. Polymorphisms in the Trace Amine Receptor 4 (TRAR4) Gene on Chromosome 6q23.2 Are Associated with Susceptibility to Schizophrenia. *Am.J.Hum.Genet.* 2004; **75**: 624-638.

Dubrana, K., Perrod, S., and Gasser, S.M. Turning telomeres off and on. *Curr Opin Cell Biol* 2001; **13**: 281-289.

Dunham, M.A., Neumann, A.A., Fasching, C.L., and Reddel, R.R. Telomere maintenance by recombination in human cells. *Nat Genet* 2000; **26**: 447-450.

Eikelboom, J.W., Lonn, E., Genest, J., Jr., Hankey, G., and Yusuf, S. Homocyst(e)ine and cardiovascular disease: a critical review of the epidemiologic evidence. *Ann Intern.Med* 1999; **131**: 363-375.

Endler, G., Klimesch, A., Sunder-Plassmann, H., Schillinger, M., Exner, M., Mannhalter, C., Jordanova, N., Christ, G., Thalhammer, R., Huber, K., and Sunder-Plassmann, R. Mean platelet volume is an independent risk factor for myocardial infarction but not for coronary artery disease. *British Journal of Haematology.* 2002; **117**: 399-404.

Erikssen, J., Hellem, A., and Stormorken, H. Chronic effect of smoking on platelet count and "platelet adhesiveness" in presumably healthy middle-aged men. *Thrombosis & Haemostasis* 1977; **38**: 606-11.

Eriksson, J.G. Early growth and coronary heart disease in later life: longitudinal study. *Br.Med.J* 2001; **322**: 949-953.

Eriksson, J., Forsen, T., Osmond, C., and Barker, D. Obesity from cradle to grave. *Int.J Obes.Relat Metab Disord.* 2003a; **27**: 722-727.

Eriksson, J. G., Forsen, T., Tuomilehto, J., Osmond, C., and Barker, D. J. Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. *Diabetologia* 2003b; **46**: 190-194.

Eriksson, J. G., Lindi, V., Uusitupa, M., Forsen, T.J., Laakso, M., Osmond, C., Barker, D.J. The effects of the Pro12Ala polymorphism of the peroxisome proliferator-activated receptor-γ2 gene on insulin sensitivity and insulin metabolism interact with size at birth. *Diabetes* 2003; **51**: 2321-2324.

Failla, G. The aging process and cancerogenesis. *Ann N Y Acad Sci* 1958; **71**: 1124-1140.

Fei, H., Berliner, J. A., Parhami, F., and Drake, T. A. Regulation of endothelial cell tissue factor expression by minimally oxidized LDL and lipopolysaccharide. *Arterioscler Thromb* 1993; **13**: 1711-1717.

Ferlicot, S., Youssef, N., Feneux, D., Delhommeau, F., Paradis, V. and Bedossa, P. Measurement of telomere length on tissue sections using quantitative fluorescence in situ hybridization (Q-FISH). *J Pathol* 2003; **5**: 661-666.

Fichtlscherer, S., Rosenberger, G., Walter, D.H., Breuer, S., Dimmeler, S., and Zeiher, A.M. Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. *Circulation* 2000; **102**: 1000-1006.

Finch, C.E. and Tanzi, R.E. Genetics of aging. *Science* 1997; **278**: 407-411.

Fisher, R.A. The correlation between relatives on the supposition of Mendalian inheritence. *Trans.R.Soc.Edinburgh* 1918; **52**: 399-433.

Frankel, S., Elwood, P., Sweetnam, P., Yarnell, J., Smith, G.D. Birthweight, body-mass index in middle age, and incident coronary heart disease. *Lancet* 2003; **348**: 1478-1480.

Frenck, R.W., Blackburn, E.H., and Shannon, K.M. The rate of telomere sequence loss in human leukocytes varies with age. *Proc.Natl.Acad.Sci.USA.* 1998; **95**: 5607-5610.

Friedlander, Y., Kidron, M., Caslake, M., Lamb, T., McConnell, M., and Bar-On, H. Low density lipoprotein particle size and risk factors of insulin resistance syndrome. *Atherosclerosis* 2000; **148**: 141-149.

Friedman, G.D., Petitti, D.B., Bawol, R.D., and Siegelaub, A.B. Mortality in cigarette smokers and quitters. Effect of base-line differences. *N Engl.J Med* 1981; **304**: 1407-1410.

Friedrich, U., Schwab, M., Griese, E.U., Fritz, P., and Klotz, U. Telomeres in neonates: new insights in fetal hematopoiesis. *Pediatr Res* 2001; 49: 252-256.

Frosst, P., Blom, H.J. and Milos, R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.* 1995; **10:**111–113.

Galis, Z S., Sukhova, G.K., Kranzhofer, R., Clark, S., and Libby, P. Macrophage foam cells from experimental atheroma constitutively produce matrix-degrading proteinases. *Proc.Natl.Acad Sci U.S.A* 1995; **92**: 402-406.

Gallagher, P.E., Li, P., Lenhart, J.R., Chappell, M.C., and Brosnihan, K.B. Estrogen regulation of angiotensin-converting enzyme mRNA. *Hypertension* 1999; **33**: 323-328.

Gao, J., Chen, D., Tian, Y., Zhang, J., and Cai, K. Effect of estrogen on telomerase activity in human breast cancer cells. *J.Huazhong.Univ Sci.Technolog.Med.Sci.* 2003; **23**: 286-7, 293.

Geer, J.C., McGill, H.C., and Strong, J.P. The fine structure of human atherosclerotic lesions. *Am.J.Pathol.* 1961; **38**: 263-287.

Gehring, K., Leroy, J.L., and Gueron, M. A tetrameric DNA structure with protonated cytosine.cytosine base pairs. *Nature* 1993; **363**: 561-565.

Gerrity, R.G. The Role of the Monocyte in Atherogenesis I. Transition of Blood-Borne Monocytes into Foam Cells in Fatty Lesions. *American Journal of Pathology* 1981a; **103**: 181-190.

Gill, P., Jeffreys, A.J., and Werrett, D.J. Forensic application of DNA 'fingerprints'. *Nature* 1985; **318**: 577-579.

Goldstein, J.L., Hobbs, H.H., Brown, M.S. Familial Hypercholesterolaemia. In: Scriver, C.R., Sly, W.S., Childs, B. et al (eds). The metabolic and molecular basis of inherited disease. New York, McGraw-Hill Companies Inc, 2001: 2863-914.

Goll, M.G. and Bestor, T.H. Histone modification and replacement in chromatin activation. *Genes Dev* 2002; **16**: 1739-1742.

Golubovskaya, V.M., Filatov, L.V., Behe, C.I., Presnell, S.C., Hooth, M.J., Smith, G.J., and Kaufmann, W.K. Telomere shortening, telomerase expression, and chromosome

instability in rat hepatic epithelial stem-like cells. *Molecular Carcinogenesis* 1999; **24**: 209-217.

Goring, H.H.H. and Ott, J. Relationship estimation in affected sib pair analysis of late-onset diseases. *Eur.J.Hum.Genet.* 1977; **5**: 69-77.

Graakjaer, J., Bischoff, C., Korsholm, L., Holstebroe, S., Vach, W., Bohr, V.A., Christensen, K., and Kolvraa, S. The pattern of chromosome-specific variations in telomere length in humans is determined by inherited, telomere-near factors and is maintained throughout life. *Mech Ageing Dev* 2003; **124**: 629-640.

Graakjaer, J., Pascoe, L., Der-Sarkissian, H., Thomas, G., Kolvraa, S., Christensen, K., and Londono-Vallejo, J. A. The relative lengths of individual telomeres are defined in the zygote and strictly maintained during life. *Aging Cell* 2004; **3**: 97-102.

Greider, C.W., and Blackburn, E.H. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 1985; **43**: 405-13.

Greider, C.W., and Blackburn, E.H. A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 1989; **337**: 331-337.

Griffith, J.D., Comeau, L., Rosenfield, S., Stansel, R.M., Bianchi, A., de Moss, H., and Lange, T. Mammalian telomeres end in a large duplex loop. *Cell* 1999; **97**: 503-514.

Gruchala, M., Ciecwierz, D., Wasag, B., Targonski, R., Dubaniewicz, W., Nowak, A., Sobiczewski, W., Ochman, K., Romanowski, P., Limon, J., and Rynkiewicz, A. Association of the ScaI atrial natriuretic peptide gene polymorphism with nonfatal myocardial infarction and extent of coronary artery disease. *American Heart Journal.* 2003; **145**: 125-131.

Gu, L., Okada, Y., Clinton, S.K., Gerard, C., Sukhova, G.K., Libby, P., and Rollins, B.J. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol.Cell* 1998; **2**: 275-281.

Hakim, A.A., Curb, J.D., Petrovitch, H., Rodriguez, B. L., Yano, K., Ross, G. W., White, L. R., and Abbott, R. D. Effects of walking on coronary heart disease in elderly men: the Honolulu Heart Program. *Circulation* 1999; **100**: 9-13.

Hansen, L. K., Grimm, R. H., Jr., and Neaton, J. D. The relationship of white blood cell count to other cardiovascular risk factors. *International Journal of Epidemiology.* 1990; **19**: 881-888.

Harle-Bachor, C. and Boukamp, P. Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. *Proc.Natl.Acad Sci U.S.A* 1996; **93**: 6476-6481.

Harley, C.B. Telomere loss: mitotic clock or genetic time bomb? *Mutat.Res* 1991; **256**: 271-282.

Harley, C.B., Vaziri, H., Counter, C. M., and Allsopp, R. C. The telomere hypothesis of cellular aging. *Exp.Gerontol.* 1992; **27**: 375-382.

Harley, C.B., Futcher, A.B., and Greider, C.W. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990; **345**: 458-460.

Harman, D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 1956; **11**: 298-300.

Harman, D. The aging process. *Proc Natl Acad Sci U S A* 1981; **78**: 7124-7128.

Harrison, D. Oxidative stress and coronary artery disease. *Can.J Cardiol.* 1998; **14 Suppl D**: 30D-32D.

Hart, R.W. and Setlow, R.B. Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. *Proc Natl Acad Sci U S A* 1974; **71**: 2169-2173.

Haseman, J.K. and Elston, R.C. The investigation of linkage between a quantitative trait and a marker locus. *Behav.Genet* 1972; **2**: 3-19.

Hastie, N.D., Dempster, M., Dunlop, M.G., Thompson, A.M., Green, D.K., and Allshire, R.C. Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 1990; **346**: 866-868.

Hastings, R., Qureshi, M., Verma, R., Lacy, P.S., and Williams, B. Telomere attrition and accumulation of senescent cells in cultured human endothelial cells. *Cell Prolif.* 2004; **37**: 317-324.

Hayflick, L. and Moorhead, P. The serial cultivation of human diploid cell strains. *Exp.Cell.Res.* 1961; **25**: 585-621.

Hayflick, L. The limited in vitro lifetime of human diploid cell strains. *Exp.Cell.Res.* 1965; **37**: 614-36.

Hayflick, L. The future of ageing. *Nature* 2000; **408**: 267-269.

He, J., Vupputuri, S., Allen, K., Prerost, M. R., Hughes, J., and Whelton, P. K. Passive smoking and the risk of coronary heart disease--a meta-analysis of epidemiologic studies.[comment]. *New England Journal of Medicine.* 1999; **340**: 920-926.

Heinrich, J., Balleisen, L., Schulte, H., Assmann, G., and van de, Loo J. Fibrinogen and factor VII in the prediction of coronary risk. Results from the PROCAM study in healthy men.[erratum appears in Arterioscler Thromb 1994 Aug;14(8):1392]. *Arteriosclerosis & Thrombosis.* 1994; **14**: 54-59.

Henderson, E.L., Geng, Y.J., Sukhova, G.K., Whittemore, A.D., Knox, J., and Libby, P. Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms. *Circulation* 1999; **99**: 96-104.

Henshaw, P.S.., Riley, E.F., and Stapleton, G.E. The biological effects of pile radiation. *Radiology* 1947; **49**: 349-364.

Henson, J.D., Neumann A.A., Yeager T.R., and Reddel R.R. Alternative lengthening of telomeres in mammalian cells. *Oncogene* 2002; **21**: 598-610.

Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., and Yamakido, M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol.* 1995; **155**: 3711-3715.

Hiyama, E., Gollahon, L., Kataoka, T., Kuroi, K., Yokoyama, T., Gazdar, AF, Hiyama, K., Piatzszek, MA, and Shay, JW. Telomerase activity in human breast tumors. *J.Natl.Cancer Inst.* 1996; **88**: 116-122.

Hornsby, P.J., Aldern, K.A., and Harris, S.E. Clonal variation in response to adrenocorticotropin in cultured bovine adrenocortical cells: relationship to senescence. *J Cell Physiol* 1986; **129**: 395-402.

Hsu, H.L., Gilley, D., Blackburn, E.H., and Chen, D.J. Ku is associated with the telomere in mammals. *Proc.Natl.Acad Sci U.S.A* 1999; **96**: 12454-12458.

Huang, Z, Wang, C, Yip, P, Yang, C, and Lee, T In Hypercholesterolemia, Lower Peripheral Monocyte Count Is Unique Among the Major Predictors of Atherosclerosis. *Art.Thromb.Vasc.Bio.* 1995; **16**: 256-261.

Hultdin, M., Gronlund, E., Norrback, K-F, Eriksson-Lindstrom, E., Just, T., and Roos, G. Telomere analysis by fluorescence in situ hybridization and flow cytometry. *Nucleic Acids Research* 1998; **26**: 3651-3656.

Humphries, S.E., Martin, S., Cooper, J., and Miller, G. Interaction between smoking and the stromelysin-1 (MMP3) gene 5A/6A promoter polymorphism and risk of coronary heart disease in healthy men. *Annals of Human Genetics.* 2002; **66**: 5-6.

Ilveskoski, E., Perola, M., Lehtimaki, T., Laippala, P., Savolainen, V., Pajarinen, J., Penttila, A., Lalu, K. H., Mannikko, A., Liesto, K. K., Koivula, T., and Karhunen, P. J. Age-dependent association of apolipoprotein E genotype with coronary and aortic atherosclerosis in middle-aged men: an autopsy study. *Circulation.* 1999; **100**: 608-613.

Imanishi, T., Hano, T., Sawamura, T., and Nishio, I. Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clin.Exp.Pharmacol.Physiol* 2004; **31**: 407-413.

Ishida, S., Hamasaki, S., Kamekou, M., Yoshitama, T., Nakano, F., Yoshikawa, A., Kataoka, T., Saihara, K., Minagoe, S., and Tei, C. Advancing age is associated with diminished vascular remodeling and impaired vasodilation in resistance coronary arteries. *Coron.Artery Dis.* 2003; **14**: 443-449.

Itahana, K., Dimri, G., and Campisi, J. Regulation of cellular senescence by p53. [Review]. *European Journal of Biochemistry* 2001; **268**: 2784-91.

Iwai, C., Akita, H., Kanazawa, K., Shiga, N., Terashima, M., Matsuda, Y., Takai, E., Miyamoto, Y., Shimizu, M., Kajiya, T., Hayashi, T., and Yokoyama, M. Arg389Gly polymorphism of the human beta1-adrenergic receptor in patients with nonfatal acute myocardial infarction. *American Heart Journal.* 2003; **146**: 106-109.

Izem, L, Rassart, E Kamate L, Falstrault, L, Rhainds, D, and Brissette, L Effect of reduced low-density lipoprotein receptor level on HepG2 cell cholesterol metabolism. *Biochem.J.* 1998; **329**: 81-88.

Jeanclos, E., Krolewski, A., Skurnick, J., Kimura, M., Aviv, H., Warram, JH, and Aviv, H. Shortened Telomere Length in White Blood Cells of Patients with IDDM. *Diabetes* 1998; **47**: 482-486.

Jeanclos, E., Schork, N.J., Kyvik, K., Kimura, M., Skurnick, J.H., and Aviv, A. Telomere length inveresly correlates with pulse pressure and is highly familial. Hypertension 2000; 36: 195-200.

Jee, S.H., Beaty, T.H., Suh, I., Yoon, Y.S. and Appel, L.J. The methylenetetrahydrofolate reductase gene is associated with increased cardiovascular risk in Japan, but not in other populations. Atherosclerosis 2000; **153:**161–168.

Jeffreys, A. J., Wilson, V., Neumann, R., and Keyte, J. Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells. *Nucleic Acids Res* 1988; **16**: 10953-10971.

Jennings, B.J., Ozanne, S.E., Dorling, M.W., and Hales, C.N. Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Lett* 1999; **448**: 4-8.

Jin, P., Gu, Y., and Morgan, D. O. Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J Cell Biol* 1996; **134**: 963-970.

Johnstone, M. T., Mittleman, M., Tofler, G., and Muller, J. E. The pathophysiology of the onset of morning cardiovascular events. *Am.J.Hypertens.* 1996; **9**: 22S-28S.

Kaartinen, M., Penttila, A., and Kovanen, P. T. Mast cells in rupture-prone areas of human coronary atheromas produce and store TNF-alpha. *Circulation* 1996; **94**: 2787-2792.

Kannel, W.B. Prevalence and natural history of electrocardiographic left ventricular hypertrophy. *Am J Med* 1983; **75**: 4-11.

Kaplan, K.M. Clinical Hypertension. 1998.

Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999; **283**: 1321-1325.

Karlseder, J., Smogorzewska, A., and de Lange, T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002; **295**: 2446-2449.

Karlseder, J. Telomere repeat binding factors: keeping the ends in check. *Cancer Lett* 2003; **194**: 189-197.

Kereiakes, D.J. The Emperor's clothes: in search of the vulnerable plaque. *Circulation* 2003; **107**: 2076-2077.

Khalil, A., Kumar, D., and Venkatesan, M. Platelet aggregation and lipid profile in offsprings of young ischemics. *Indian Pediatrics.* 1997; **34**: 16-19.

Kim, H.C., Hwang, Y.K., Choi, M.T., Shin, Y.W. and Hong, Y.S. The methylenetetrahydrofolate reductase gene polymorphism in Koreans with coronary heart disease. Int. J. Cardiol. 2001; **78:**13–17.

Kim, S.H., Han, S., You, Y.H., Chen, D.J., and Campisi, J. The human telomere-associated protein TIN2 stimulates interactions between telomeric DNA tracts in vitro. *EMBO Rep.* 2003; **4**: 685-691.

Kim, S.H., Kaminker, P., and Campisi, J. TIN2, a new regulator of telomere length in human cells. *Nat Genet* 1999; **23**: 405-412.

Kinouchi, Y., Hiwatashi, N., Chida, M., Nagashima, F., Takagi, S., Maekawa, H., and Toyota, T. Telomere shortening in the colonic mucosa of patients with ulcerative colitis. *Journal of Gastroenterology.* 1998; **33**: 343-348.

Kirkwood, T. B. Evolution of ageing. *Nature* 1977; **270**: 301-304.

Kishi, S., Wulf, G., Nakamura, M., and Lu, KP Telomeric protein Pin2/TRF1 induces mitotic entry and apoptosis in cells with short telomeres and is down-regulated in human breast tumors. *Oncogene* 2001; **20**: 1497-508.

Klerk, M. et al. The MTHFR Studies Collaboration Group, MTHFR 677 C→T polymorphism and risk of coronary heart disease. JAMA 2002; **288:** 2023–2031.

Klimov, A.N., Gurevich, V.S., Nikiforova, A.A., Sahtilina, L.V., Kuzmin, A.A., Plavinsky, S.L., and Teryukova, N.P. Antioxidant activity of high-density lipoproteins in vivo. Atherosclerosis 1993; 100: 13-18.

Kluijtmans, L.A.J. and Whitehead, A.S. Methylenetetrahydrofolate reductase genotypes and predisposition to atherothrombotic disease. Eur. Heart J. 2001; **22:**294–299.

Koba, S., Hirano, T., Sakaue, T., Sakai, K., Kondo, T., Yorozuya, M., Suzuki, H., Murakami, M., and Katagiri, T. Role of small dense low-density lipoprotein in coronary artery disease patients with normal plasma cholesterol levels. [Japanese]. *Journal of Cardiology - Supplement.* 2000; **36**: 371-378.

Koering, C.E., Pollice, A., Zibella, M.P., Bauwens, S., Puisieux, A., Brunori, M., Brun, C., Martins, L., Sabatier, L., Pulitzer, J.F., and Gilson, E. Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity. *EMBO Rep* 2002; 3: 1055-1061.

Kol, A., Bourcier, T., Lichtman, A. H., and Libby, P. Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. *J Clin.Invest* 1999; **103**: 571-577.

Kong, A., Gudbjartsson, D. F., Sainz, J., Jonsdottir, G. M., Gudjonsson, S. A., Richardsson, B., Sigurdardottir, S., Barnard, J., Hallbeck, B., Masson, G., Shlien, A., Palsson, S. T., Frigge, M. L., Thorgeirsson, T. E., Gulcher, J. R., and Stefansson, K. A high-resolution recombination map of the human genome. *Nat Genet* 2002; **31**: 241-247.

Kornet, L., Lambregts, J., Hoeks, A. P., and Reneman, R. S. Differences in near-wall shear rate in the carotid artery within subjects are associated with different intima-media thicknesses. *Arterioscler Thromb Vasc Biol* 1998; **18**: 1877-1884.

Korpelainen, H. Genetic maternal effects on human life span through the inheritance of mitochondrial DNA. *Hum.Hered.* 1999; **49**: 183-185.

Kozik, A., Bradbury, E.M., and Zalensky, A. Increased telomere size in sperm cells of mammals with long terminal (TTAGGG)n arrays. *Molecular Reproduction & Development* 1998; **51**: 98-104.

Kruk, P.A., Rampino, N.J., and Bohr, V.A. DNA damage and repair in telomeres: Relation to aging. *Proc.Natl.Acad.Sci.USA.* 1995; **92**: 258-262.

Kubar, A., Yapar, M., Besirbellioglu, B., Avci, I. Y., and Guney, C. Rapid and quantitative detection of mumps virus RNA by one-step real-time RT-PCR. *Diagn.Microbiol Infect.Dis.* 2004; **49**: 83-88.

Kubes, P., Suzuki, M., and Granger, D. N. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 1991; **88**: 4651-4655.

Kuller, L.H. and Sutton-Tyrrell, K. Aging and cardiovascular disease. Use of subclinical measurements. *Cardiol.Clin.* 1999; **17**: 51-65, viii.

Kunz, J. and Keim, U. On the regeneration of aortic endothelium at different ages. *Mech Ageing Dev* 1975; **4**: 361-369.

Kuro-o, M., Matsumara, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., Kaname, T., Kume, E., Iwasaki, H., Lida, A., Shiraki-Lida, T., Nishikawa, S., Nagai, R., and Nabeshima, Y. Mutation in the mouse klotho gene leads to a syndrome resemling ageing. *Nature* 1997; **390**: 45-51.

Kyo, S., Takakura, M., Kanaya, T., Zhuo, W., Fujimoto, K., Nishio, Y., Orimo, A., and Inoue, M. Estrogen activates telomerase. *Cancer Res* 1999; **59**: 5917-5921.

LaFleur, D.W., Chiang, J., Fagin, J.A., Schwartz, S. M., Shah, P. K., Wallner, K., Forrester, J. S., and Sharifi, B. G. Aortic smooth muscle cells interact with tenascin-C through its fibrinogen-like domain. *J Biol Chem.* 1997; **272**: 32798-32803.

Landmark, K. Smoking and coronary heart disease. [Review]. *Tidsskrift for Den Norske Laegeforening* 2001; **121**: 1710-2.

Lansdorp, P.M., Verwoerd, N.P., van de Rijke, F.M., Dragowska, V., Little, M-T., Dirks, R.W., Raap, A.K., and Tanke, H.J. Heterogeneity in telomere length of human chromosomes. *Human Molecular Genetics* 1996; **5**: 685-691.

Latham, R.D., Westerhof, N., Sipkema, P., Rubal, B.J., Reuderink, P., and Murgo, J.P. Regional wave travel and reflections along the human aorta: a study with six simultaneous micromanometric pressures. *Circulation* 1985; **72**: 1257-1269.

Latron, Y., Chautan, M., Anfosso, F., Alessi, M. C., Nalbone, G., Lafont, H., and Juhan-Vague, I. Stimulating effect of oxidized low density lipoproteins on plasminogen activator inhibitor-1 synthesis by endothelial cells. *Arterioscler Thromb* 1991; **11**: 1821-1829.

Laurent, S., Boutouyrie, P., Asmar, R., Gautier, I., Laloux, B., Guize, L., Ducimetiere, P., and Benetos, A. Aortic stiffness is an independent predictor of all-cause and cardiovascular mortality in hypertensive patients. *Hypertension* 2001; **37**: 1236-1241.

Lehto, S, Niskanen, L, Suhonen, M, Ronnema, T, Seikku, P, and Laakso, M Association between Chlamydia pneumoniae and intimal calcification in femoral arteries of nondiabetic patients. *Archives of Internal Medicine* 2002; **162**: 594-599.

Leinonen, M., and Saikku, P. Evidence for infectious agents in cardiovascular disease and atherosclerosis. *The Lancet Infectious Diseases* 2002; **2**: 11-17.

Leon, A.S. and Connett, J. Physical activity and 10.5 year mortality in the Multiple Risk Factor Intervention Trial (MRFIT). *Int.J Epidemiol.* 1991; **20**: 690-697.

Leon, D., Lithell, H.O., Vagero, D., Koupilova, I., Mohsen, R., Berglund, L., Lithell, U.B., and McKeigue, P.M. Reduced fetal growth rate and increased risk of death from ischaemic heart disease: a cohort study of 15000 Swedish men and women born 1915-29. *Br.Med.J* 1998; **317**: 241-245.

Lerman, A. and Burnett, J.C., Jr. Intact and altered endothelium in regulation of vasomotion. *Circulation* 1992; **86**: III12-III19.

Leteurtre, F., Li, X., Guardiola, P., Le Roux, G., Sergere, J. C., Richard, P., Carosella, E.D., and Gluckman, E. Accelerated telomere shortening and telomerase activation in Fanconi's anaemia. *British Journal of Haematology.* 1999; **105**: 883-893.

Leung, D.Y., Glagov, S., and Mathews, M.B. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. *Science* 1976; **191**: 475-477.

Li, A., Tedde, R., Krozowski, Z.S., Pala, A., Li, K.X., Shackleton, C.H., Mantero, F., Palermo, M., and Stewart, P.M. Molecular basis for hypertension in the "type II variant" of apparent mineralocorticoid excess. *Am J Hum Genet* 1998; **63**: 370-379.

Libby, P., and Aikawa, M. Mechanisms of plaque stabilization with statins. *Am J Cardiol* 2003; **91**: 4B-8B.

Libby, P. Molecular bases of the acute coronary syndromes. *Circulation* 1995; **91**: 2844-2850.

Libby, P., Geng, Y. J., Aikawa, M., Schoenbeck, U., Mach, F., Clinton, S. K., Sukhova, G. K., and Lee, R. T. Macrophages and atherosclerotic plaque stability. *Curr.Opin.Lipidol.* 1996; **7**: 330-335.

Libby, P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* 2001; **104**: 365-372.

Libby, P., Ridker, P.M., and Maseri, A. Inflammation and atherosclerosis. *Circulation* 2002; **105**: 1135-1143.

Liddle, G., Bledsoe, T. and Coppage, W.A. familial renal disorder stimulating primary aldosteronism but with negligible aldosterone secretion. *Transanctions of the Association of American Physicans* 1963; **76**: 199-213.

Lingner, J., Hughes, T.R., Shevchenko, A., Mann, M., Lundblad, V., and Cech, T.R. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997; **276**: 561-567.

Lintula, S., Vesalainen, S., Rannikko, A., Zhang, W. M., Finne, P., Stenman, J., and Stenman, U. H. Quantification of prostate specific antigen mRNA levels in circulation after prostatic surgery and endocrine treatment by quantitative reverse transcription-polymerase chain reaction. *Scand.J Clin.Lab Invest* 2004; **64**: 93-100.

Lipid Research Clinics Program Lipid and lipoprotein analysis. 2004;

Liu, W. and Saint, D.A. Validation of a quantitative method for real time PCR kinetics. *Biochem.Biophys.Res Commun.* 2002; **294**: 347-353.

Liuzzo, G, Biasucci, LM, Gallimore, JR, Grillo, RL, Rebuzzi, AG, Pepys, MB, and Maseri, A The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *New Engl.J.Med.* 1994; **331**: 417-24-

Livak, K.J. *ABI Prism 7700 Sequence Detection System. User Bulletin no. 2.* 1997.

Livak, K.J., and Schmittgen, T.W. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-Ct\ method.}$ *Methods* 2001; **25**: 402-408.

Llevadot, J., Murasawa, S., Kureishi, Y., Uchida, S., Masuda, H., Kawamoto, A., Walsh, K., Isner, J. M., and Asahara, T. HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells. *J Clin.Invest* 2001; **108**: 399-405.

Ludmer, P.L., Selwyn, A. P., Shook, T. L., Wayne, R. R., Mudge, G. H., Alexander, R. W., and Ganz, P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl.J Med* 1986; **315**: 1046-1051.

Lusis, A.J. Atherosclerosis. *Nature* 2000; **407**: 233-241.

Lyons, R.J., Deane, R., Lynch, D.K., Ye, Z.S., Sanderson, G.M., Eyre, H.J., Sutherland, G.R., and Daly, R.J. Identification of a novel human tankyrase through its interaction with the adaptor protein Grb14. *Journal of Biological Chemistry* 2001; **276**: 17172-80.

Mabuchi, A., Haga, N., Maeda, K., Nakashima, E., Manabe, N., Hiraoka, H., Kitoh, H., Kosaki, R., Nishimura, G., Ohashi, H., and Ikegawa, S. Novel and recurrent mutations clustered in the von Willebrand factor A domain of MATN3 in multiple epiphyseal dysplasia. *Hum.Mutat.* 2004; **24**: 439-440.

Mach, F., Sauty, A., Iarossi, A.S., Sukhova, G. K., Neote, K., Libby, P., and Luster, A. D. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin.Invest* 1999; **104**: 1041-1050.

Mahoney, E.M., Khoo, J.C., and Steinberg, D. *Proc.Natl.Acad.Sci.USA* 1982; **79**: 1639-1642.

Maier, J.A., Voulalas, P., Roeder, D., and Maciag, T. Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. *Science* 1990; **249**: 1570-1574.

Maier, J.A., Statuto, M., and Ragnotti, G. Senescence stimulates U937-endothelial cell interactions. *Exp.Cell Res* 1993; **208**: 270-274.

Makarov, V.L., Hirose, Y., and Langmore, J.P. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell* 1997; **88**: 657-666.

Manhem, K., Ahlm, H., Milsom, I., and Svensson, A. Transdermal oestrogen reduces daytime blood pressure in hypertensive women. *J Hum.Hypertens.* 1998; **12**: 323-327.

Manson, J.E., Hu, F.B., Rich-Edwards, J.W., Colditz, G.A., Stampfer, M.J., Willett, W.C., Speizer, F.E., and Hennekens, C.H. A prospective study of walking as compared with vigorous exercise in the prevention of coronary heart disease in women. *N Engl.J Med* 1999; **341**: 650-658.

Manttari, M., Tenkanen, L., Alikoski, T., and Manninen, V. Alcohol and coronary heart disease: the roles of HDL-cholesterol and smoking. *Journal of Internal Medicine.* 1997; **241**: 157-163.

Marenberg, M.E., Risch, N., Berkman, L.F., Floderus, B., and de Faire, U. Genetic susceptibility to death from coronary heart disease in a study of twins. *N Engl.J Med* 1994; **330**: 1041-1046.

Martens, U.M., Zijlmans, J.M., Poon, S.S.S., Dragowska, W., Yui, J., Chavez, E.A., Ward, R.K., and Lansdorp, P.M. Short telomeres on human chromosome 17p. *Nat.Genet.* 1998; **18**: 76-80.

Martens, U.M., Brass, V., Ehgelhardt, M., Glaser, S., Waller, C.F., Lange, W., Schmoor, C., Poon, SS, and Lansdrop, PM Measurement of telomere length in haematopoietic cells using in situ hybridization techniques. *Biochem.Soc.Trans.* 2000; **28**: 245-50.

Martin, G.M. Genetic syndromes in man with potential relevance to the pathobiology of aging. *Birth Defects Orig.Artic.Ser.* 1978; **14**: 5-39.

Maseri, A. and Fuster, V. Is there a vulnerable plaque? *Circulation* 2003; **107**: 2068-2071.

McClintock, B. The production of homozygous deficient tissues with mutant characteristics by means of the aberrant mitotic behavior of ring-shaped chromosomes. *Genetics* 1938; **23**: 315-376.

McClintock, B. The behavior in successive nuclear divisions of a chromosome broken at meiosis. *Proceedings of the National Academy of Sciences* 1939; **25**: 405-416.

McClintock, B. The stability of broken ends of chromosomes in Zea mays. *Genetics* 1941; **26**: 234-282.

McCully, K.S. and Wilson, R.B. Homocysteine theory of arteriosclerosis. *Atherosclerosis* 1975; **22** : 215-227.

McElligott, R., and Wellinger, R.J. The terminal DNA structure of mammalian chromosomes. *EMBO Journal* 1997; **16**: 3705-3714.

Melk, A., Ramassar, V., Helms, LM, Moore, R., Rayner, D., Solez, K., and Halloran, PF Telomere shortening in kidneys with age. *Journal of the American Society of Nephrology* 2000; **11**: 444-53.

Mendelsohn, M.E. and Karas, R.H. The protective effects of estrogen on the cardiovascular system. *N Engl.J Med* 1999; **340**: 1801-1811.

Metcalfe, J.A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P.J., and Taylor, A.M. Accelerated telomere shortening in ataxia telangiectasia. *Nat Genet* 1996; **13**: 350-353.

Miller, G.J. High density lipoproteins and atherosclerosis. *Ann.Rev.Med.* 1980; **31**: 97-108.

Minamino, T., Miyauchi, H., Yoshida, T., Ishida, Y., Yoshida, H., and Komuro, I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* 2002; **105**: 1541-1544.

Mitchell, J.R., Wood, E., and Collins, K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999; **402**: 551-555.

Mittleman, M.A., Maclure, M., Tofler, G.H., Sherwood, J.B., Goldberg, R.J., and Muller, J.E. Triggering of acute myocardial infarction by heavy physical exertion. Protection against triggering by regular exertion. Determinants of Myocardial Infarction Onset Study Investigators. *N Engl.J Med* 1993; **329**: 1677-1683.

Mohaghegh, P., Karow, J.K., Brosh, Jr RM, Jr., Bohr, V.A., and Hickson, I.D. The Bloom's and Werner's syndrome proteins are DNA structure-specific helicases. *Nucleic Acids Res* 2001a; **29**: 2843-2849.

Mohaghegh, P. and Hickson, I. D. DNA helicase deficiencies associated with cancer predisposition and premature ageing disorders. *Hum.Mol.Genet.* 2001b; **10**: 741-746.

Morita, H., Taguchi, J. and Kurihara, H. Genetic polymorphism of 5,10-methylenetetrahydrofolate reductase as a risk factor for coronary artery disease. Circulation 1997; **95:**2032–2036.

Mueller, S.N., Rosen, E.M., and Levine, E.M. Cellular senescence in a cloned strain of bovine fetal aortic endothelial cells. *Science* 1980; **207**: 889-891.

Muir, K.W., Weir, C.J., Alwan, W., Sqiure, I.B., and Lees. K.R. C-reactive protein and outcome after ischemic stroke. *Stroke* 1999; **30**: 981-985.

Mukhopadhyay, N., Almasy, L., Schroeder, M., Mulvihill, W.P., and Weeks, D.E. Mega2, a data-handling program for facilitating genetic linkage and association analyses. *Am.J.Hum.Genet.* 1999; **65**: A436-

Muller, H.J. *Collect.Net.* 1938; **13**: 181-98.

Muller, J.E. and Tofler, G.H. Triggering and hourly variation of onset of arterial thrombosis. *Ann.Epidemiol.* 1992; **2**: 393-405.

Mulvany, M.J. and Aalkjaer, C. Structure and function of small arteries. *Physiol Rev* 1990; **70**: 921-961.

Mune, T., Rogerson, F.M., Nikkila, H., Agarwal, A.K., and White, P.C. Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nature Genetics* 1995; **10**: 394-399.

Nakamura, K., Izumiyama-Shimomura, N., Sawabe, M., Arai, T., Aoyagi, Y., Fujiwara, M., Tsuchiya, E., Kobayashi, Y., Kato, M., Oshimura, M., Sasajima, K., Nakachi, K., and Takubo, K. Comparative analysis of telomere lengths and erosion with age in human epidermis and lingual epithelium. *J Invest Dermatol* 2002; **119**: 1014-1019.

Nakamura, M., Zhou, X. Z., Kishi, S., Kosugi, I., Tsutsui, Y., and Lu, K. P. A specific interaction between the telomeric protein Pin2/TRF1 and the mitotic spindle. *Curr.Biol* 2001; **11**: 1512-1516.

Nakamura, M., Zhou, X.Z., Kishi, S., and Lu, K.P. Involvement of the telomeric protein Pin2/TRF1 in the regulation of the mitotic spindle. *FEBS Lett.* 2002; **514**: 193-198.

Nakamura, T.M., Morin, G.B., Chapman, K.B., Weinrich, S.L., Andrews, W.H., Lingner, J., Harley, C.B., and Cech, T.R. Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997; **277**: 955-959.

National Research Council, 1989.

Nawrot, T.S., Staessen, J.A., Gardner, J.P., and Aviv, A. Telomere length and possible link to X chromosome. *Lancet* 2004b; **363**: 507-510.

Newbold, R.F., Overell, R.W., and Connell, J.R. Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. *Nature* 1982; **299**: 633-635.

Nichols, W.W., and O'Rourke, M. McDonald's Blood Flow in Arteries: Theoretical, Experimental and Clinical Principles. 1998; **4th**: 54-401.

Nigam, A., Mitchell, G. F., Lambert, J., and Tardif, J. C. Relation between conduit vessel stiffness (assessed by tonometry) and endothelial function (assessed by flow-mediated

dilatation) in patients with and without coronary heart disease. *Am.J Cardiol.* 2003; **92**: 395-399.

Ning, Y., Xu, J.F., Li, Y., Chavez, L., Riethman, H.C., Lansdorp, P.M., and Weng, N.P. Telomere length and the expression of natural telomeric genes in human fibroblasts. *Hum Mol Genet* 2003; **12**: 1329-1336.

Nogues, X., Senti, M., Pedro-Botet, J., Molina, L., Serrat, R., Pons, S., and Rubies-Prat, J. [Coronary heart disease and lipoprotein (a): relationship with other lipid cardiovascular risk factors]. [Spanish]. *Medicina Clinica.* 1992; **98**: 171-174.

O'Connell, J.R. and Weeks, D.E. PedCheck: A program for identifying marker typing incompatibilities in linakge analysis. *Am.J.Hum.Genet.* 1988; **10**: A288-

O'Leary, D.H., Polak, J.F., Kronmal, R.A., Manolio, T.A., Burke, G.L., and Wolfson, S.K., Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular Health Study Collaborative Research Group. *N Engl.J Med* 1999; **340**: 14-22.

O'Sullivan, J.N., Bronner, M.P., Brentnall, T.A., Finley, J.C., Shen, W.T., Emerson, S., Emond, M. J., Gollahon, K.A., Moskovitz, A.H., Crispin, D.A., Potter, J.D., and Rabinovitch, P.S. Chromosomal instability in ulcerative colitis is related to telomere shortening. *Nature Genetics.* 2002; **32**: 280-284.

Ogami, M., Ikura, Y., Ohsawa, M., Matsuo, T., Kayo, S., Yoshimi, N., Hai, E., Shirai, N., Ehara, S., Komatsu, R., Naruko, T., and Ueda, M. Telomere Shortening in Human Coronary Artery Diseases. *Arterioscler Thromb Vasc Biol* 2004;

Ohyashiki, JH, Hayashi, S., Yahata, N., Iwama, H., Ando, K., Tauchi, T., and Ohyashiki, K. Impaired telomere regulation mechanism by TRF1 (telomere-binding protein), but not TRF2 expression, in acute leukemia. *International Journal of Oncology* 2001; **18**: 593-8.

Okuda, K., Bardeguez, A., Gardner, J.P., Rodriguez, P., Ganesh, V., Kimura, M., Skurnick, J., Awad, G., and Aviv, A. Telomere length in the newborn. *Pediatr Res* 2002; **52**: 377-381.

Okuda, K., Khan, M.Y., Skurnick, J., Kimura, M., Aviv, H., and Aviv, A. Telomere attrition of the human abdominal aorta: relationships with age and atherosclerosis. *Atherosclerosis* 2003; **152**: 391-398.

Okumura, K., Matsui, H., Ogawa, Y., Takahashi, R., Matsubara, K., Imai, H., Imamura, A., Mizuno, T., Tsuzuki, M., and Kitamura, Y. The polymorphism of the beta3-adrenergic receptor gene is associated with reduced low-density lipoprotein particle size. *Metabolism: Clinical & Experimental.* 2003; **52**: 356-361.

Olovnikov, A.M. A Theory of Marginotomy. *J.Theor.Biol.* 1973; **41**: 181-190.

Olsen, E.G.J. Ischemic Disease of the Myocardium and Its Complications. 1991; **2**: 673-

Omvik, P. How smoking affects blood pressure. [Review] [69 refs]. *Blood Pressure.* 1996; **5**: 71-77.

Opresko, P.L., von Kobbe, C., Laine, J.P., Harrigan, J., Hickson, I.D., and Bohr, V.A. Telomere-binding protein TRF2 binds to and stimulates the Werner and Bloom syndrome helicases. *J Biol Chem.* 2002; **277**: 41110-41119.

Opresko, P.L., Otterlei, M., Graakjaer, J., Bruheim, P., Dawut, L., Kolvraa, S., May, A., Seidman, M. M., and Bohr, V. A. The Werner Syndrome Helicase and Exonuclease Cooperate to Resolve Telomeric D Loops in a Manner Regulated by TRF1 and TRF2. *Mol.Cell* 2004; **14**: 763-774.

Orgel, L.E. The maintenance of the accuracy of protein synthesis and its relevance to ageing. *Proc Natl Acad Sci U S A* 1963; **49**: 517-521.

Packard, C.J., O'Reilly, D.S., Caslake, M.J., McMahon, A.D., Ford, I., Cooney, J., Macphee, C.H., Suckling, K.E., Krishna, M., Wilkinson, F.E., Rumley, A., and Lowe, G.D. Lipoprotein-associated phospholipase A2 as an independent predictor of coronary heart disease. West of Scotland Coronary Prevention Study Group. *N Engl.J Med* 2000; **343**: 1148-1155.

Paneth, N. and Susser, M. Early origin of coronary heart disease (the "Barker hypothesis"). *BMJ* 1995; **310**: 411-412.

Pasceri, V. and Yeh, E.T. A tale of two diseases: atherosclerosis and rheumatoid arthritis. *Circulation* 1999; **100**: 2124-2126.

Pasceri, V., Willerson, J.T., and Yeh, E.T. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 2000; **102**: 2165-2168.

Pasceri, V., Cheng, J.S., Willerson, J. T., Yeh, E.T., and Chang, J. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation* 2001; **103**: 2531-2534.

Pasterkamp, G., Schoneveld, A.H., van der Wal, A.C., Hijnen, D.J., van Wolveren, W.J., Plomp, S., Teepen, H. L., and Borst, C. Inflammation of the atherosclerotic cap and shoulder of the plaque is a common and locally observed feature in unruptured plaques of femoral and coronary arteries. *Arterioscler Thromb Vasc Biol* 1999; **19**: 54-58.

Pawelec, G., Effros, R.B., Caruso, C., Remarque, E., Barnett, Y., and Solana, R.T. cells and aging (update february 1999). *Front Biosci.* 1999; **4**: D216-D269.

Perls, T., Levenson, R., Regan, M., and Puca, A. What does it take to live to 100? *Mech Ageing Dev* 2002; **123**: 231-242.

Perls, T.T., Alpert, L., and Fretts, R.C. Middle-aged mothers live longer. *Nature* 1997; **389**: 133-

Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001; **29**: e45.

Phan, A.T. and Mergny, J.L. Human telomeric DNA: G-quadruplex, i-motif and Watson-Crick double helix. *Nucleic Acids Res* 2002; **30**: 4618-4625.

Phipps, K., Barker, D.J., Hales, C.N., Fall, C.H., Osmond, C., and Clark, P.M. Fetal growth and impaired glucose tolerance in men and women. *Diabetologia* 1993; **36**: 225-228.

Pietila, K., Harmoinen, A., Poyhonen, L., Koskinen, M., Heikkila, J., and Ruosteenoja, R. Intravenous streptokinase treatment and serum C-reactive protein in patients with acute myocardial infarction. *Br.Heart J* 1987; **58**: 225-229.

Pietila, K.O., Harmoinen, A.P., Jokiniitty, J., and Pasternack, A.I. Serum C-reactive protein concentration in acute myocardial infarction and its relationship to mortality during 24 months of follow-up in patients under thrombolytic treatment. *Eur.Heart J* 1996; **17** : 1345-1349.

Pitsavos, C., Panagiotakos, D.B., Chrysohoou, C., Tzioumis, K., Papaioannou, I., Stefanadis, C., and Toutouzas, P. Association between passive cigarette smoking and the risk of developing acute coronary syndromes: the CARDIO2000 study. *Heart & Vessels.* 2002; **16**: 127-130.

Ponten, J., Stein, W. D., and Shall, S. A quantitative analysis of the aging of human glial cells in culture. *J Cell Physiol* 1983; **117**: 342-352.

Prasad, A., Zhu, J., Halcox, J.P., Waclawiw, M.A., Epstein, S.E., and Quyyumi, A.A. Predisposition to atherosclerosis by infections: role of endothelial dysfunction. *Circulation* 2002; **106**: 184-190.

Qiao, J.H., Tripathi, J., Mishra, N.K., Cai, Y., Tripathi, S., Wang, X.P., Imes, S., Fishbein, M.C., Clinton, S.K., Libby, P., Lusis, A. J., and Rajavashisth, T. B. Role of macrophage colony-stimulating factor in atherosclerosis: studies of osteopetrotic mice. *Am.J Pathol* 1997; **150**: 1687-1699.

Raftopoulos, C., Bermingham, M.A., and Steinbeck, K.S. Coronary heart disease risk factors in male adolescents, with particular reference to smoking and blood lipids. *Journal of Adolescent Health.* 1999; **25**: 68-74.

Rajavashisth, T.B., Xu, X.P., Jovinge, S., Meisel, S., Xu, X. O., Chai, N.N., Fishbein, M.C., Kaul, S., Cercek, B., Sharifi, B., and Shah, P. K. Membrane type 1 matrix metalloproteinase expression in human atherosclerotic plaques: evidence for activation by proinflammatory mediators. *Circulation* 1999; **99**: 3103-3109.

Renauld, H., Aparicio O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev* 1993; **7**: 1133-1145.

Retterstol, L., Eikvar, L., Bohn, M., Bakken, A., Erikssen, J., and Berg, K. C-reactive protein predicts death in patients with previous premature myocardial infarction - A 10 year follow-up study. *Atherosclerosis* 2002; **160**: 433-440.

Rheinwald, J.G. and Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; **6**: 331-343.

Rhind, N., Furnari, B., and Russell, P. Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes Dev* 1997; **11**: 504-511.

Rich-Edwards, J.W. Stampfer, M.J., Manson, J.E., Rosner, B., Hankinson, S.E., Colditz, G.A., Willett, W.C., and Hennekens, C.H.. Birthweight and risk of cardiovascular disease in a cohort of women followed up since 1976. *Br.Med.J* 1997; **315**: 396-400.

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P., and Hennekens, C.H. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl.J Med* 1997; **336**: 973-979.

Rimm, E.B., Stampfer, M.J., Giovannucci, E., Ascherio, A., Spiegelman, D., Colditz, G.A., and Willett, W.C. Body size and fat distribution as predictors of coronary heart disease among middle-aged and older US men. *Am.J Epidemiol.* 1995; **141**: 1117-1127.

Rittling, S.R., Brooks, K.M., Cristofalo, V.J., and Baserga, R. Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts. *Proc.Natl.Acad Sci U.S.A* 1986; **83**: 3316-3320.

Rodriguez-Pla, A., Bosch-Gil, J. A., Echevarria-Mayo, J. E., Rossello-Urgell, J., Solans-Laque, R., Huguet-Redecilla, P., Stone, J. H., and Vilardell-Tarres, M. No detection of parvovirus B19 or herpesvirus DNA in giant cell arteritis. *J.Clin.Virol.* 2004; **31** : 11-15.

Rosenberg, L., Kaufman, D.W., Helmrich, S.P., and Shapiro, S. The risk of myocardial infarction after quitting smoking in men under 55 years of age. *N Engl.J Med* 1985; **313**: 1511-1514.

Rosenberg, L., Palmer, J.R., and Shapiro, S. Decline in the risk of myocardial infarction among women who stop smoking. *N Engl.J Med* 1990; **322**: 213-217.

Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 1993; **362**: 801-809.

Ross, R. Atherosclerosis - an inflammatory disease. *New Engl.J.Med.* 1999; **340**: 115-126.

Rotenbacher, D., Fischer, G.H., Hoffmeister, A., Hoffmann, M.M., März, W., Bode G. et al. Homocysteine and methylenetetrahydrofolate reductase genotype: association with risk of coronary heart disease and relation to inflammatory, haemostatic, and lipid parameters. Atherosclerosis 2002; **162:**193–200.

Rubio, M.A., Kim, S.H., and Campisi, J. Reversible manipulation of telomerase expression and telomere length. Implications for the ionizing radiation response and replicative senescence of human cells. *J Biol Chem* 2002; **277**: 28609-28617.

Sadoshima, J., Jahn, L., Takahashi, T., Kulik, T. J., and Izumo, S. Molecular characterization of the stretch-induced adaptation of cultured cardiac cells. An in vitro model of load-induced cardiac hypertrophy. *J Biol Chem.* 1992; **267**: 10551-10560.

Safar, M.E. Systolic blood pressure, pulse pressure and arterial stiffness as cardiovascular risk factors. *Curr.Opin.Nephrol.Hypertens.* 2001; **10**: 257-261.

Sager, R. Senescence as a mode of tumor suppression. *Environ.Health Perspect.* 1991; **93**: 59-62.

Salk, D. Werner's syndrome: a review of recent research with an analysis of connective tissue metabolism, growth control of cultured cells, and chromosomal aberrations. *Hum.Genet.* 1982; **62**: 1-5.

Samani, N.J., Boultby, R., Butler, R., Thompson, JR, and Goodhall, AH Telomere shortening in atherosclerosis. *Lancet* 2001; **358**: 472-73.

Samper, E., Goytisolo, F. A., Slijepcevic, P., van Buul, P. P., and Blasco, M. A. Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Rep.* 2000; **1**: 244-252.

Saren, P., Welgus, H. G., and Kovanen, P. T. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J Immunol.* 1996; **157**: 4159-4165.

Saretzki, G., Sitte, N., Merkel, U., Wurm, RE, and von Zglinicki, T. Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. *Oncogene* 1999; **18**: 5148-5158.

Sawchuk, A.P., Unthank, J.L., and Dalsing, M.C. Drag reducing polymers may decrease atherosclerosis by increasing shear in areas normally exposed to low shear stress. *J.Vasc.Surg.* 1999; **30**: 761-764.

Schachter, F., Faure-Delanef, L., Guenot, F., Rouger, H., Froguel, P., Lesueur-Ginot, L., and Cohen, D. Genetic associations with human longevity at the APOE and ACE loci. *Nat Genet* 1994; **6**: 29-32.

Schaefer, E.J., Lamon-Fava, S., Jenner, J.L., McNamara, J.R., Ordovas, J.M., Davis, C.E., Abolafia, J.M., Lippel, K., and Levy, R.I. Lipoprotein(a) levels and risk of coronary heart disease in men. The lipid Research Clinics Coronary Primary Prevention Trial.[comment]. *JAMA.* 1994; **271**: 999-1003.

Schaetzlein, S., Lucas-Hahn, A., Lemme, E., Kues, W. A., Dorsch, M., Manns, M. P., Niemann, H., and Rudolph, K. L. Telomere length is reset during early mammalian embryogenesis. *Proc.Natl.Acad.Sci.U.S.A* 2004; **101**: 8034-8038.

Schaffitzel, C., Berger, I., Postberg, J., Hanes, J., Lipps, H. J., and Pluckthun, A. In vitro generated antibodies specific for telomeric guanine-quadruplex DNA react with Stylonychia lemnae macronuclei. *Proc Natl Acad Sci U S A* 2001; **98**: 8572-8577.

Schmidt, A.M., and Stern, D. Atherosclerosis and diabetes: the RAGE connection. *Curr.Atheroscler.Rep.* 2000; **2**: 430-436.

Schneider, D.J., Hayes, M., Wadsworth, M., Taatjes, H., Rincon, M., Taatjes, D. J., and Sobel, B. E. Attenuation of neointimal vascular smooth muscle cellularity in atheroma by plasminogen activator inhibitor type 1 (PAI-1). *J.Histochem.Cytochem.* 2004; **52**: 1091-1099.

Schulz, V.P., Zakian, V.A., Ogburn, C.E., McKay, J., Jarzebowicz, A.A., Edland, S.D., and Martin, G.M. Accelerated loss of telomeric repeats may not explain accelerated replicative decline of Werner syndrome cells. *Hum.Genet* 1996a; **97**: 750-754.

Sen, D. and Gilbert, W. Formation of parallel four-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature* 1988; **334**: 364-366.

Shaper, A.G. and Wannamethee, S.G. Alcohol intake and mortality in middle aged men with diagnosed coronary heart disease. *Heart (British Cardiac Society).* 2000; **83**: 394-399.

Shay, J.W., Wright, W.E., and Werbin, H. Defining the molecular mechanisms of human cell immortalization. *Biochim.Biophys.Acta* 1991; **1072**: 1-7.

Shay, J.W. and Bacchetti, S. *Eur.J.Cancer* 1997; **33**: 787-791.

Shay, J.W., and Wright, W.E. Hayflick, his limit, and cellular ageing. *Nature Reviews Molecular Cell Biology* 2000; **1**: 72-6.

Shen, M., Haggblom, C., Vogt, M., Hunter, T., and Lu, K. P. Characterization and cell cycle regulation of the related human telomeric proteins Pin2 and TRF1 suggest a role in mitosis. *Proc.Natl.Acad Sci U.S.A* 1997; **94**: 13618-13623.

Shepherd, J. The West of Scotland Coronary Prevention Study: a trial of cholesterol reduction in Scottish men. *Am.J Cardiol.* 1995; **76**: 113C-117C.

Shepherd, J., Cobbe, S. M., Ford, I., Isles, C. G., Lorimer, A. R., MacFarlane, P. W., McKillop, J. H., and Packard, C. J. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl.J Med* 1995; **333**: 1301-1307.

Sillesen, H., and Nielsen, T. Clinical significance of intraplaque hemorrhage in carotid artery disease. *J Neuroimaging* 1998; **8**: 15-19.

Singer, M.S., and Gottschling, D.E. TLC1: template RNA component of Saccharomyces cerevisiae telomerase. *Science* 1994; **266**: 404-409.

Slagboom, P.E., Droog, S., and Boomsma, D.I. Genetic Determination of Telomere Size in Humans: A Twin Study of Three Age Groups. *Am.J.Hum.Genet.* 1994b; **55**: 876-882.

Smith, J.D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J., and Miyata, M. Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc.Natl.Acad Sci U.S.A* 1995; **92**: 8264-8268.

Smith, S., Giriat, I., and de Lange, T. Tankyrase, a poly (ADP-ribose) polymerase at human telomeres. *Science* 1998; **282**: 1484-7.

Smith, S. and de Lange, T. Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes. *Journal of Cell Sciences* 1999; **112**: 3649-56.

Smith, S. and de Lange, T. Tankyrase promotes telomere elongation in human cells. *Current Biology* 2000; **10**: 1299-302.

Smogorzewska, A. and de Lange, T. Different telomere damage signaling pathways in human and mouse cells. *EMBO J* 2002; **21**: 4338-4348.

Smogorzewska, A., Karlseder, J., Holtgreve-Grez, H., Jauch, A., and de Lange, T. DNA ligase IV-dependent NHEJ of deprotected mammalian telomeres in G1 and G2. *Curr.Biol* 2002; **12**: 1635-1644.

Smucker, E.J. and Turchi, J.J. TRF1 inhibits telomere C-strand DNA synthesis in vitro. *Biochemistry* 2001; **40**: 2426-32.

Sorensen, K.E., Celermajer, D.S., Georgakopoulos, D., Hatcher, G., Betteridge, D. J., and Deanfield, J. E. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin.Invest* 1994; **93**: 50-55.

Southern, E.M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J.Mol.Biol.* 1975; **98**: 503-517.

Stansel, R.M., de Lange, T., and Griffith, J.D. T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *EMBO J* 2001; **20**: 5532-5540.

Stanulis-Praeger, B. Cellular Senescence revisited: a review. *Mech.Ageing Dev.* 1987; 38: 1-48.

Stavropoulos, D.J., Bradshaw, P.S., Li, X., Pasic, I., Truong, K., Ikura, M., Ungrin, M., and Meyn, M.S. The Bloom syndrome helicase BLM interacts with TRF2 in ALT cells and promotes telomeric DNA synthesis. *Hum.Mol.Genet* 2002; **11**: 3135-3144.

Stein, C.E., Fall, C.H., Kumaran, K., Osmond, C., Cox, V., Barker, D.J. Fetal growth and coronary heart disease in South India. *Lancet* 1996; **348**: 1269-1273.

Stemme, S., Faber, B., Holm, J., Wiklund, O., Witztum, J.L., and Hansson, G.K. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc.Natl.Acad Sci U.S.A* 1995; **92**: 3893-3897.

Stuhlinger, M.C., Oka, R.K., Graf, E.E., Schmolzer, I., Upson, B.M., Kapoor, O., Szuba, A., Malinow, M.R., Wascher, T.C., Pachinger, O., and Cooke, J.P. Endothelial dysfunction induced by hyperhomocyst(e)inemia: role of asymmetric dimethylarginine. *Circulation* 2003; **108**: 933-938.

Sukhova, G.K., Schonbeck, U., Rabkin, E., Schoen, F.J., Poole, A. R., Billinghurst, R. C., and Libby, P. Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation* 1999; **99**: 2503-2509.

Sundquist, W.I. and Klug, A. Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops. *Nature* 1989; **342**: 825-829.

Szilard, L. On the nature of the ageing process. *Proc Natl Acad Sci U S A* 1959; **45**: 35-45.

Tanaka, H., Dinenno, F.A., Monahan, K.D., DeSouza, C.A., and Seals, D.R. Carotid artery wall hypertrophy with age is related to local systolic blood pressure in healthy men. *Arterioscler Thromb Vasc Biol* 2001; **21**: 82-87.

Tanaka, M., Kyo, S., Takakura, M., Kanaya, T., Sagawa, T., Yamashita, K., Okada, Y., Hiyama, E., and Inoue, M. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. *Am.J Pathol* 1998; **153**: 1985-1991.

Tanasescu, M. and Hu, F.B. Alcohol consumption and risk of coronary heart disease among individuals with type 2 diabetes. [Review] [44 refs]. *Current Diabetes Reports.* 2001; **1**: 187-191.

Tassin, J., Malaise, E., and Courtois, Y. Human lens cells have an in vitro proliferative capacity inversely proportional to the donor age. *Exp.Cell Res* 1979; **123**: 388-392.

Tewari, D., Kim, H., Feria, W., Russo, B., and Acland, H. Detection of West Nile virus using formalin fixed paraffin embedded tissues in crows and horses: quantification of viral transcripts by real-time RT-PCR. *J Clin.Virol.* 2004; **30**: 320-325.

Tham, W.H. and Zakian, V.A. Transcriptional silencing at Saccharomyces telomeres: implications for other organisms. *Oncogene* 2002; **21**: 512-21.

The Homocysteine Studies Collaboration Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA* 2002; **288**: 2015-2022.

The WOSCOPS Study Group Screening experience and baseline characteristics in the West of Scotland Coronary Prevention Study. *Am J Cardiol* 1995; **76**: 485-491.

Theegarten, D., Anhenn, O., Hotzel, H., Wagner, M., Marra, A., Stamatis, G., Mogilevski, G., and Sachse, K. A comparative ultrastructural and molecular biological study on Chlamydia psittaci infection in alpha-1 antitrypsin deficiency and non-alpha-1 antitrypsin deficiency emphysema versus lung tissue of patients with hamartochondroma. *BMC.Infect.Dis.* 2004; **4**: 38-

Tice, R.R., Schneider, E.L., Kram, D., and Thorne, P. Cytokinetic analysis of the impaired proliferative response of peripheral lymphocytes from aged humans to phytohemagglutinin. *J Exp.Med* 1979; **149**: 1029-1041.

Topper, J.N., Cai, J., Falb, D., and Gimbrone, M.A., Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc.Natl.Acad Sci U.S.A* 1996; **93**: 10417-10422.

Torzewski, M., Rist, C., Mortensen, R. F., Zwaka, T. P., Bienek, M., Waltenberger, J., Koenig, W., Schmitz, G., Hombach, V., and Torzewski, J. C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol* 2000; **20**: 2094-2099.

Toschi, V., Gallo, R., Lettino, M., Fallon, J.T., Gertz, S.D., Fernandez-Ortiz, A., Chesebro, J.H., Badimon, L., Nemerson, Y., Fuster, V., and Badimon, J.J. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 1997; **95**: 594-599.

Troen, B.R. The biology of aging. *Mt.Sinai J Med* 2003; **70**: 3-22.

Ueland, P.M., Refsum, H., Beresford, S.A., and Vollset, S. E. The controversy over homocysteine and cardiovascular risk. *Am.J Clin.Nutr.* 2000; **72**: 324-332.

Ulaner, G.A., and Giudice, L.C. Developmental regulation of telomerase activity in human fetal tissues during gestation. *Mol Hum Reprod* 1997; **3**: 769-773.

Valmadrid, C.T., Klein, R., Moss, S.E., Klein, B.E., and Cruickshanks, K.J. Alcohol intake and the risk of coronary heart disease mortality in persons with older-onset diabetes mellitus. *JAMA.* 1999; **282**: 239-246.

van de, Wiel A., van Golde, P.M., Kraaijenhagen, R.J., dem Borne, P.A., Bouma, B.N., and Hart, H.C. Acute inhibitory effect of alcohol on fibrinolysis. *European Journal of Clinical Investigation.* 2001; **31**: 164-170.

van der Wal, A.C., Becker, A.E., van der Loos, C.M., and Das, P.K. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 1994; **89**: 36-44.

van Steensel, B., Smogorzewska, A., and de Lange, T. TRF2 protects human telomeres from end-to-end fusions. *Cell* 1998; **92**: 401-13.

Vasa, M., Breitschopf, K., Zeiher, A.M., and Dimmeler, S. Nitric Oxide Activates Telomerase and Delays Endothelial Cell Senescence. *Circ.Res.* 2000; **87**: 540-542.

Vasa, M., Fichtlscherer, S., Adler, K., Aicher, A., Martin, H., Zeiher, A.M., and Dimmeler, S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 2001a; **103**: 2885-2890.

Vasa, M., Fichtlscherer, S., Aicher, A., Adler, K., Urbich, C., Martin, H., Zeiher, A.M., and Dimmeler, S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ.Res* 2001b; **89**: E1-E7.

Vashisht, S., Wasir, H., and Srivastava, L.M. Association between incidence of lipoprotein(a) positivity and coronary heart disease. *Indian Heart Journal.* 1992; **44**: 223-226.

Vaziri, H., Schachter, F., Uchida, I., Wei, L., Zhu, X., Effros, R., Cohen, D., and Harley, C.B. Loss of Telomeric DNA during Aging of Normal and Trisomy 21 Human Lymphocytes. *Am.J.Hum.Genet.* 1993; **52**: 661-667.

Vaziri, H. Critical telomere shortening regulated by the ataxia-telangiectasia gene acts as a DNA damage signal leading to activation of p53 protein and limited life-span of human diploid fibroblasts. A review. *Biochemistry (Mosc.)* 1997; **62**: 1306-1310.

Vaziri, H., West, M.D., Allsopp, R.C., Davison, T.S., Wu, Y.S., Arrowsmith, C.H., Poirier, G.G., and Benchimol, S. ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose) polymerase. *EMBO J* 1997; **16**: 6018-6033.

Vendrell, J., Fernandez-Real, J. M., Gutierrez, C., Zamora, A., Simon, I., Bardaji, A., Ricart, W., and Richart, C. A polymorphism in the promoter of the tumor necrosis factor-alpha gene (-308) is associated with coronary heart disease in type 2 diabetic patients. *Atherosclerosis.* 2003; **167**: 257-264.

Viitanen, L., Pihlajamaki, J., Miettinen, R., Karkkainen, P., Vauhkonen, I., Halonen, P., Kareinen, A., Lehto, S., and Laakso, M. Apolipoprotein E gene promoter (-219G/T)

polymorphism is associated with premature coronary heart disease. *Journal of Molecular Medicine.* 2001; **79**: 732-737.

Visser, M., Bouter, L.M., McQuillan, G M., Wener, M.H., and Harris, T.B. Elevated C-reactive protein levels in overweight and obese adults. *JAMA* 1999; **282**: 2131-2135.

Vita, J. A., Treasure, C.B., Nabel, E. G., McLenachan, J.M., Fish, R.D., Yeung, A.C., Vekshtein, V. I., Selwyn, A.P., and Ganz, P. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation* 1990; **81**: 491-497.

Volpe, M., Musumeci, B., De Paolis, P., Savoia, C., and Morganti, A. Angiotensin II AT2 receptor subtype: an uprising frontier in cardiovascular disease? *J Hypertens.* 2003; **21**: 1429-1443.

von Zglinicki, T. Role of oxidative stress in telomere length regulation and replicative senescence. *Annals of the New York Academy of Sciences* 2000a; **908**: 99-110.

von Zglinicki, T., Serra, V., Lorenz, M., Saretzki, G., Lenzen-Grossimlighaus, R., Gessner, R., Risch, A., and Steinhagen-Thiessen, E. Short telomeres in patients with vascular dementia: an indicator of low antioxidative capacity and a possible risk factor? *Laboratory Investigation.* 2000b; **80**: 1739-1747.

von Zglinicki, T. Oxidative stress shortens telomeres. *Trends Biochem Sci* 2002; **27**: 339-344.

Wallner, K., Li, C., Shah, P.K., Fishbein, M.C., Forrester, J.S., Kaul, S., and Sharifi, B.G. Tenascin-C is expressed in macrophage-rich human coronary atherosclerotic plaque. *Circulation* 1999; **99**: 1284-1289.

Walter, D.H., Rittig, K., Bahlmann, F.H., Kirchmair, R., Silver, M., Murayama, T., Nishimura, H., Losordo, D.W., Asahara, T., and Isner, J.M. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation* 2002; **105**: 3017-3024.

Wang, Z., Huang, Y., Zou, J., Cao, K., Xu, Y., and Wu, J.M. Effects of red wine and wine polyphenol resveratrol on platelet aggregation in vivo and in vitro. *International Journal of Molecular Medicine.* 2002; **9**: 77-79.

Watanabe, N. Telomerase, cell immortality and cancer. [Review]. *Hokkaido Journal of Medical Science* 2001; **76**: 127-32.

Westendorp, R.G. and Kirkwood, T.B. Human longevity at the cost of reproductive success. *Nature* 1998; **396**: 743-746.

Wiemann, S.U., Satyanarayana, A., Tsahuridu, M., Tillmann, H.L., Zender, L., Klempnauer, J., Flemming, P., Franco, S., Blasco, M.A., Manns, M.P., and Rudolph, K.L. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB Journal.* 2002; **16**: 935-942.

Willett, W.C., Hennekens, C.H., Bain, C., Rosner, B., and Speizer, F.E. Cigarette smoking and non-fatal myocardial infarction in women. *Am.J Epidemiol.* 1981; **113**: 575-582.

Williams, C.D., Boggess, J.F., LaMarque, L.R., Meyer, W.R., Murray, M.J., Fritz, M.A., and Lessey, B.A.A. prospective, randomized study of endometrial telomerase during the menstrual cycle. *J.Clin.Endocrinol.Metab* 2001; **86**: 3912-3917.

Williams, W.C. Pleiotropy, natural selection and the evolution of senescence. *Evolution* 1957; **11**: 398-411.

Willich, S.N., Lewis, M., Lowel, H., Arntz, H.R., Schubert, F., and Schroder, R. Physical exertion as a trigger of acute myocardial infarction. Triggers and Mechanisms of Myocardial Infarction Study Group. *N Engl.J Med* 1993; **329**: 1684-1690.

Wilson, P.W., Schaefer, E.J., Larson, M.G. et al. Apolipoprotein E alleles and risk of coronary disease. A meta-analysis. Arterio.Thromb.Vasc.Biol. 1996; 16: 1250-5.

Wilson, R.C., Harbison, M.D., Krozowski, Z.S., Funder, J.W., Shackleton, C.H., Hanauske-Abel, H.M., Wei, J.Q., Hertecant, J., Moran, A., Neiberger, R.E., and *et al.* Several homozygous mutations in the gene for 11 beta-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *J Clin Endocrinol Metab* 1995; **80**: 3145-3150.

Witztum, J.L. and Berliner, J.A. Oxidized phospholipids and isoprostanes in atherosclerosis. *Curr.Opin.Lipidol.* 1998; **9**: 441-448.

Wood, J.G. and Sinclair, D.A. TPE or not TPE? It's no longer a question. *Trends Pharmacol Sci* 2002; **23**: 1-4.

Wright, W.E., and Shay, J.W. Telomere positional effects and the regulation of cellular senescence. *Trends Genet* 1992; **8**: 193-197.

Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W., and Shay, J.W. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet* 1996; **18**: 173-179.

Wyllie, F.S., Jones, C.J., Skinner, J.W., Haughton, M.F., Wallis, C., Wynford-Thomas, D., Faragher, R.G., and Kipling, D. Telomerase prevents the accelerated cell ageing of Werner syndrome fibroblasts. *Nat Genet* 2000a; **24**: 16-17.

Wynn, R.F., Cross, M.A., Hatton, C., Will, A.M., Lahford, L.S., Dexter, T.M., and Testa, N.G. Accelerated telomere shortening in young recipients of allogenic bone-marrow transplants. *The Lancet* 1998; **351**: 178-181.

Xiao, N.M., Zhang, Y.M., Zheng, Q., and Gu, J. Klotho is a serum factor related to human aging. *Chin Med J (Engl.)* 2004; **117**: 742-747.

Xu, D., Neville, R., and Finkel, T. Homocysteine accelerates endothelial cell senescence. *FEBS Letters* 2000; **470**: 20-24.

Xu, X. P., Meisel, S. R., Ong, J. M., Kaul, S., Cercek, B., Rajavashisth, T. B., Sharifi, B., and Shah, P. K. Oxidized low-density lipoprotein regulates matrix metalloproteinase-9 and its tissue inhibitor in human monocyte-derived macrophages. *Circulation* 1999; **99**: 993-998.

Yamamoto, K., Ikeda, U., and Shimada, K. Role of mechanical stress in monocytes/macrophages: implications for atherosclerosis. *Curr. Vasc. Pharmacol.* 2003; **1**: 315-319.

Yokoya, K., Takatsu, H., Suzuki, T., Hosokawa, H., Ojio, S., Matsubara, T., Tanaka, T., Watanabe, S., Morita, N., Nishigaki, K., Takemura, G., Noda, T., Minatoguchi, S., and Fujiwara, H. Process of progression of coronary artery lesions from mild or moderate stenosis to moderate or severe stenosis: A study based on four serial coronary arteriograms per year. *Circulation* 1999; **100**: 903-909.

Yoshimura, T., Hisatomi, A., Kajihara, S., Yasutake, T., Ogawa, Y., Mizuta, T., Ozaki, I., Utsunomiyai, T., and Yamamoto, K. The relationship between insulin resistance and polymorphisms of the endothelial nitric oxide synthase gene in patients with coronary artery disease. *Journal of Atherosclerosis & Thrombosis.* 2003; **10**: 43-47.

Youngren, K., Jeanclos, E., Aviv, H., Kimura, M., Stock, J., Hanna, M., Skurnick, J., Bardeguez, A., and Aviv, A. Synchrony in telomere length of the human fetus. *Hum Genet* 1998; **102**: 640-643.

Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G.M., Mulligan, J., and Schellenberg, G.D. Positional cloning of the Werner's syndrome gene. *Science* 1996a; **272**: 258-262.

Zeichner, S.L., Palumbo, P., Feng, Y., Xiao, X., Gee, D., Sleasman, J., Goodenow, M., Biggar, R., and Dimitrov, D. Rapid telomere shortening in children. *Blood* 1999; **93**: 2824-2830.

Zhou, B.S., and Elledge, S.J. The DNA damage response: putting checkpoints in perspective. *Nature* 2000; **408**: 433-439.

Zhou, X.Z., Perrem, K., and Lu, K.P. Role of Pin2/TRF1 in telomere maintenance and cell cycle control. *J Cell Biochem.* 2003; **89**: 19-37.

Zhu L, Hathcock KS, Hande P, Lansdorp PM, Seldin MF, and Hodes RJ Telomere length regulation in mice is linked to a novel chromosome locus. *Proc Natl Acad Sci U S A* 1998; **95**: 8648-8653.

Zito, F., Lowe, G.D., Rumley, A., McMahon, A.D., Humphries, S.E., and WOSCOPS Study Group West of Scotland Coronary Prevention Study Association of the factor XII 46C>T polymorphism with risk of coronary heart disease (CHD) in the WOSCOPS study. *Atherosclerosis.* 2002; **165**: 153-158.

Zwaka, T.P., Hombach, V., and Torzewski, J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. *Circulation* 2001; **103**: 1194-1197.

SUPPLIERS

Alpha Innotech Corporation
2401 Merced St.
San Leandro
CA 94577
USA

Amersham Biosciences UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire
HP79NA

Applied Biosystems
850 Lincoln Centre Drive
Foster City
CA 94404
USA

Bioline UK Ltd
16 The Edge Business Centre
Humber Road
London
NW2 6EW

Corbett Research UK
94 The Sycamores
Milton
Cambridge
CB4 6XL

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leicestershire
LE11 5RG

ICN Pharmaceuticals Ltd
Cedarwood
Chineham Business Park
Crockford Lane
Basingstoke
RG24 8WD

Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley
UK
PA4 9RF

Promega UK Ltd

Sigma-Aldrich Company Ltd

Delta House
Chilworth Research Centre
Southampton
SO16 7NS

Fancy Road
Poole
Dorser
BH12 4QH