Telomere Biology in Ischaemic Cardiomyopathy.

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Telomere Biology in Ischaemic Cardiomyopathy

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ABSTRACT

Implantable cardioverter defibrillators (ICDs) reduce mortality in patients with ischaemic cardiomyopathy at high risk of ventricular arrhythmias (VA). However, the current indication for ICD prescription needs improvement. Telomere length and telomerase activity in leukocytes have been shown to correlate with biological aging and pathogenesis of cardiovascular diseases. Their role in arrhythmias, however, is unknown.

I examined telomere biology in ischaemic cardiomyopathy patients and established its association with VA. This study stemmed from the primary hypothesis that telomere shortening at the time of the index event (myocardial infarction), results in poor myocardial repair process and predisposes patients to greater arrhythmic tendency. Hence there would be a correlation between leukocyte telomere length, load-of-short telomeres and telomerase activity with VA occurrence in these patients. I also investigated the effect of genetic variation on telomerase activity and VA. From a basic science perspective, different mechanisms of telomere shortening were studied by using a novel method for measuring critically short telomeres.

90 ischaemic cardiomyopathy patients with primary prevention ICDs were recruited. 35 had received appropriate therapy from the ICD for potentially-fatal VA while the remaining 55 patients had not. No significant differences in baseline demographic data were seen between the two groups. There was no significant difference in the age and sex adjusted mean telomere length analysed by qPCR between the groups ($p=0.66$). In contrast, the load-of-short telomeres assessed by Universal-STELA method and telomerase activity by TRAP assay were both higher in patients who had appropriate ICD therapy and were significantly associated with incidence of ICD therapy

(p=0.02, p= 0.02). Genetic variation in telomerase activity was observed with a significant correlation between telomerase and VA in C/C genotype only.

These data collectively suggest that telomere biology is a promising area of exploration for further research in risk stratification for ICD prescription.

Statement of Originality

I, Vinit Sawhney, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Signature:

Date:

Details of collaboration and publications:

Collaborators:

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PUBLICATIONS AND PRESENTATIONS FROM THIS WORK

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Abstract Presentations

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3. Leukocyte telomere dynamics in patients with implantable cardioverter defibrillators: potential biomarkers for ventricular arrhythmias. *(Heart Rhythm 2012, PO3-82. Web link:* http://www.abstractsonline.com/Plan/ViewAbstract.aspx*)*
4. Does modification to the approach of contacting potential participants improve recruitment to clinical trials? *(Poster presentation, William Harvey Day, Barts Health NHS, London 2012)*

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ABCD	alternans before cardioverter defibrillator
AF	atrial fibrillation
AUC	area under the curves
ALIVE	azimiLide post infract survival evaluation
ATRAMI	autonomic tone and reflexes after MI
BHAT	beta-blocker heart attack trial
BRS	baroreceptor sensitivity
BNP	brain natriuretic peptide
CABG-Patch	coronary artery bypass graft patch
CAD	coronary artery disease
CARISMA	cardiac arrhythmias and risk stratification after myocardial infarction
CASS	coronary artery surgery study
CAST	cardiac arrhythmia suppression trial
COMPANION	comparison of medical therapy, pacing, and defibrillation in heart failure
CRP	C-reactive protein
CHF	congestive heart failure
CMR	cardiac MRI
CRT-D	cardiac resynchronisation therapy - defibrillator
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DIG	digoxigenin
RNA	ribonucleic acid

DEFINITE	defibrillators in non-ischaemic cardiomyopathy treatment evaluation
DINAMIT	defibrillator in acute MI trial
ELISA	enzyme linked immunosorbent assay
EMIAT	European MI amiodarone trial
FH	familial hypercholesterolemia
FDA	food and drug administration
FRET	forster type energy transfer
fQRS	fragmented QRS
FISH	fluorescent in-situ hybridisation
GFR	glomerular filtration rate
HRT	heart rate turbulence
hMSC	human mesenchymal stem cells
HRV	heart rate variability
hTERT	telomerase reverse transcriptase
ICD	implantable cardioverter defibrillator
IVCD	intraventricular conduction delay
LBBB	left bundle branch block
LDL	low density lipoprotein
LVEF	left ventricular ejection fraction
MADITI	multicenter automatic defibrillator implantation trial I
MADITII	multicenter automatic defibrillator implantation trial I
MADIT-CRT	Cardiac-resynchronisation therapy for prevention of heart failure events
MASTER	microvolt T wave alternans testing for risk stratification of post-myocardial infarction patients

MGB	minor grove binder
MI	myocardial infarction
MTWA	microvolt T-wave alternans
MPS	multicenter post infarction study
MERIT-HF	metoprolol randomised intervention trial in-congestive heart failure
MUSTT	multicentre unsustained tachycardia trial
MTP	microplate
NAF1	nuclear assembly factor 1
NSVT	non-sustained ventricular tachycardia
NTC	non-template control
NC	negative controls
NFQ	non-fluorescent quencher
NYHA	New York heart association
OBFC1	oligonucleotide/oligosaccharide-binding fold containing 1
POT1	protection of telomeres 1
QOL	quality of life
qPCR	quantitative polymerase chain reaction
RAP1	repressor/activator protein 1
ROS	reactive oxygen species
RTEL1	regulator of telomere elongation helicase 1
RCF	relative centrifugal force
RPM	revolutions per minute
S	samples
SAECG	signal averaged electrocardiogram
SCD	sudden cardiac death

SCD-HeFT	Sudden Cardiac Death in Heart Failure Trial
SNP	single nucleotide polymorphisms
TERC	telomerase RNA component
TIN2	TRF1-interacting nuclear protein 2
TPE	telomere position effect
TPP1	TIN2-interacting protein 1
TRF1	telomere repeat binding factor 1
TRF2	telomere repeat binding factor 2
TRF	telomere restriction fragment
TRAP	telomere repeat amplification protocol
TWA	T wave alternans
USTELA	universal single telomere length analysis
VLPs	ventricular late potentials
VPB	ventricular premature beat
VA	ventricular arrhythmias
VT	ventricular tachycardia
WOSCOPS	west of scotland primary prevention study
WCC	white cell count
WBCs	white blood cells

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DEDICATION

I owe a debt of gratitude to my grandfather, Dr J.S. Sawhney, for his eternal faith in my potential.

Chapter 1 – INTRODUCTION

1.1 Sudden Cardiac Death (SCD) and Ischaemic Cardiomyopathy

1.1.1 Sudden Cardiac Death

Sudden cardiac death (SCD) is defined as unexpected death from cardiac causes within one hour of symptom onset in a person with or without pre-existing heart disease. Because the duration of symptoms preceding the terminal event usually defines the sudden nature of death, World Health Organization defines SCD as unexpected death within 1 hour of symptom onset if witnessed or within 24 hours of the person having been observed alive and symptom-free if unwitnessed. Exclusion of a non-cardiac cause is critical.[1]

SCD is a major health problem affecting 50,000 – 70,000 lives in the United Kingdom and 250,000 – 300,000 in the United States every year and represents the largest proportion of deaths attributable to coronary artery disease (CAD) and caused by an arrhythmic event. [2,3] It accounts for more than 50% of all CAD deaths and 15-20% of all deaths. [4] Despite the recent advances in cardiopulmonary resuscitation and post resuscitation care, survival to hospital discharge for out of hospital cardiac arrests is approximately 5 - 8%. Moreover, the small proportion of people who do survive a cardiac arrest are at a higher risk of further arrest. Approximately 85–90% of SCD is due to the first recognised arrhythmic event; the remaining 10–15% is caused by recurrent events. [2,3] It poses a major challenge to the clinician as the vast majority of SCDs occur unwitnessed at home, a significant proportion in seemingly low risk individuals with no known cardiac disease. [3,5]

The incidence of SCD increases markedly with age, independent of gender or ethnic background. [6,7] Despite this observation, the proportion of

deaths that are sudden is larger in the younger age group with a large socioeconomic impact. Men have a three fold higher risk of SCD than women, which might be a reflection of the lower incidence of CAD in the latter. Racial differences in SCD have also been reported with the relative risk of cardiac arrest being higher in Afro-Carribeans than Caucasians. [6,7]

SCD is a complex disorder with varied etiology. It is believed to be caused by a dynamic interaction between a trigger event and underlying pathological substrate leading to a fatal ventricular arrhythmia (VA), which causes haemodynamic collapse resulting in cardiac arrest. The commonest primary electric event (contributing to the initiation of a fatal arrhythmia) recorded at the time of SCD is ventricular tachycardia (VT) in patients with CAD. [6,8]

The various underlying disease processes implicated in causality of SCD include CAD, cardiomyopathies (dilated, hypertrophic and arrhythmogenic right ventricular cardiomyopathy) and channelopathies (long QT, short QT, brugada, catecholaminergic polymorphic ventricular tachycardia). Despite the varied etiology, CAD remains the commonest substrate accounting for nearly 75% of all SCDs. [4,9,10] Hence it is one of the important subgroups to target with the aim to reduce the incidence of SCD.

1.1.2 Sudden Cardiac Death and Coronary Artery Disease

CAD is the most common and frequent cause of SCD in the Western world responsible for ~ 75% of all SCDs.[4,7] CAD can result in SCD in mainly three clinical scenarios: (1) acute myocardial infarction (MI) (2) myocardial ischaemia without infarction and (3) myocardial structural changes in the form of scarring or ventricular dilatation following prior infarction or chronic ischaemia. [4,7,9,10] Evidence from autopsy studies in sudden death victims indicates that stable

plaques and chronic atherosclerotic changes were the pre-dominant findings in the vast majority. [11]

CAD, with its current prevalence, has a significant impact on the risk of SCD. Data from the Framingham heart study show that CAD was responsible for a 2.8- to 5.3- fold increase in risk of SCD. The risk of SCD post MI has a temporal trend and is maximum in the first 30 days, remaining significant at 6 months and gradually decreasing with time with a plateau phase at two years.[12]

The mechanism underlying SCD post MI is believed to be an electrical event due to a VA triggered by an arrhythmogenic stimulus on the background of an ischaemic substrate. VT degenerating into ventricular fibrillation and then asystole is the commonest pathophysiological cascade involved in fatal arrhythmias. Bradyarrhythmias and electromechanical dissociation have been recorded at the time of SCD in patients with advanced heart failure. [6,8]

In this subset of SCD victims, VT can be triggered by acute myocardial ischaemia resulting from abrupt changes in regional myocardial blood flow due to alterations in coronary artery structure and/or function, such as spasm, platelet thrombi, dissection, or plaque rupture. Alternatively, it can be in the context of an anatomical substrate in the form of myocardial scarring in the absence of acute myocardial ischaemia. Mechanisms leading to cardiac arrest in the absence of acute ischaemia include the generation of re-entrant circuits as a result of fibrosis on the border of an infarct zone, triggered activity in scar tissue, worsening heart failure and metabolic/electrolyte disturbances. In addition, certain genetic polymorphisms may predispose individuals to a greater chance of developing VAs in the presence of an initiating insult. [13,14,15]

Preventive strategies to reduce the global burden of SCD can take the form of primary prevention of CAD by minimising the associated risk factors or primary prevention of SCD by risk stratification and intervention in those individuals with established cardiovascular disease placing them at higher risk than the general population. Although it might seem obvious that reduction of cardiovascular risk factors would result in reduction of CAD and SCD, robust evidence supporting this notion is currently lacking. [3]

For prevention of SCD in CAD patients, better identification of risk factors and pathogenesis of SCD in CAD is essential. It is critical to know who is likely to remain stable for months or years after the index event (MI) and who has a likely substrate for fatal VAs. Timely and accurate identification of individuals likely to suffer an arrhythmic event in the general population lacks a widely accepted, highly sensitive and specific predictive model. Current parameters for risk stratification of patients with CAD for SCD include medical history (presence of non-sustained ventricular tachycardia or syncope), family history of MI, New York Heart Association Class (NYHA), left ventricular ejection fraction, ECG criteria (QRS duration, QT interval, QT dispersion), signal averaged ECG, heart rate variability and baroreflex sensitivity. Candidate gene and genome wide association studies have helped identify single nucleotide polymorphisms (SNP) associated with SCD in CAD patients. However, the sensitivity and specificity of these parameters needs to be established in large-scale population studies for them to be used as independent risk predictors. Also, the heritability of SCD in this subgroup remains poorly understood. Although the above parameters have been used in combination in various risk prediction algorithms, the only one established as

an independent risk predictor of SCD in CAD is left ventricular ejection fraction (LVEF). [1]

Interventions and therapies directed towards CAD in general and SCD in particular have been tried to reduce the incidence of SCD in the general population. Pharmacological therapy including beta-blockers, anti-arrhythmics and statins have shown some potential. However, amongst all medications tested, only beta-blockers have shown definite reduction in incidence of SCD in patients who survived a MI. The Beta-Blocker Heart Attack Trial (BHAT) study showed that β-blockade with propranolol reduced all-cause mortality by 25%, especially in patients with diminished LV function and/or VAs. [16] The most effective of all interventions to date is the implantable cardioverter defibrillator (ICD). Multiple randomised control trials (RCTs) have shown a marked survival benefit with ICD therapy in patients with poor left ventricular function on the background of prior MI. In this subgroup of patients, ICD therapy has been associated with a 23%–55% mortality reduction, due almost exclusively to a reduction in SCD. [1]

1.2 Implantable Cardioverter Defibrillators (ICD) in Ischaemic Cardiomyopathy

1.2.1 Implantable Cardioverter Defibrillator – The Evolution

The implantable cardioverter defibrillator (ICD) is recognised as an effective therapeutic modality for termination of malignant VAs and hence prevention of SCD. Multiple prospective, randomised, multicenter clinical trials have documented its efficacy in improved survival by reduction of SCD in patients with CAD.

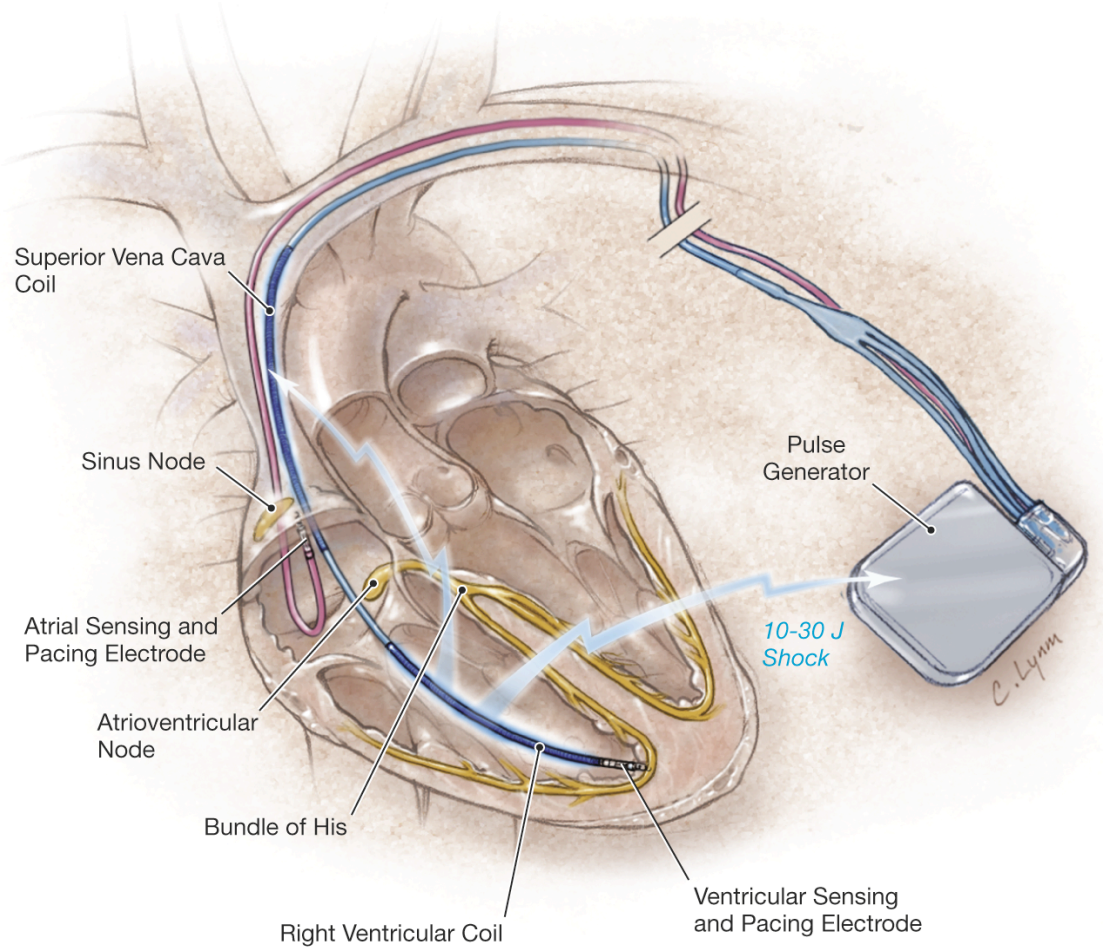

Figure 1: Image showing the implantable cardioverter defibrillator with pulse generator and atrial and ventricular leads in the heart

(Taken from JAMA. 2006;296(23):2839-2847) [17]

The concept of an ICD first emerged in late 1960s with the advent of external defibrillation and the first clinically applicable, ICD implant was performed in 1980 by Mirowski. [18] Initial clinical acceptance was slow, but improved substantially after the Food and Drug Administration (FDA) approval was granted in 1985.

Since its initial conception, the ICD has undergone phenomenal change in structure and function with advances in technology. The very early devices were short lived, could provide shock only therapy, weighed 250 g and required

thoracotomy and abdominal generator implants. The current devices have become smaller and are suitable for pre-pectoral implants. They have extended longevity, endocardial/epicardial lead systems with capacity for multi-programmable tiered therapy with advanced rhythm discrimination.

The current data supporting ICDs (primary and secondary indications) have been derived from several RCTs, the vast majority of which were published between late 1996 and early 2005, in a period of less than 10 years. The evidence from these trials started to get consideration and support from the larger scientific community after the publication of the initial results of the CAST (Cardiac Arrhythmia Suppression Trial) study in 1989. [19,20] CAST had an indispensable influence on arrhythmia management by concluding that anti-arrhythmics (flecainide/encainide) should not be used in patients with minimally symptomatic VA after MI due to increased risk of arrhythmic deaths. [19,20] The paradigm for preventive therapy of SCD drifted away from anti-arrhythmic therapy, providing a boost to the potential of device therapy. The role of ICDs in arrhythmia detection and the evidence base for the benefits of primary prevention ICDs and their efficacy in preventing SCD, especially in ischaemic cardiomyopathy, is presented next.

1.2.2 ICD as a Tool for Arrhythmia Detection and SCD prevention

The role of ICDs in SCD prevention is largely based on their ability to detect and treat arrhythmias. ICD generators have advanced programmability and internal circuitry, which is designed to deliver high-energy shocks within seconds of arrhythmia detection. They have detailed data logging capabilities and can record precise measurements of arrhythmia rates, time of occurrence and response to therapy. The stored electrograms provide beat-to-beat interval

data for VA detection and are an important diagnostic to assess the appropriateness of therapy and any malfunction of the system.

Arrhythmia detection requires effective sensing of the intrinsic cardiac activity and fulfillment of the programmed detection algorithm. The programmed algorithms help the ICD to differentiate between supraventricular and ventricular arrhythmias and hence prevent inappropriate therapy. After each delivered therapy, the ICD determines whether the tachycardia was terminated and the need for any further therapy.

Therapies delivered for VAs can be antitachycardia pacing (ATP) and shock therapy. ATP refers to high-rate, overdrive pacing to terminate VT and has shown to be more effective in terminating spontaneous VTs (slower) rather than induced VTs (faster). [21] ATP can be unsuccessful in the setting of poor LVEF and very rapid ventricular rates (>200bpm). [22] However, ATP still remains the preferred mode of initial therapy as it has >90% success rate in terminating VT, [23] is painless and contributes to preservation of battery life when compared to cardioversion. Failing ATP or as a first line therapy for VF, ICDs can deliver high-energy shocks for cardioversion. Although effective, this drains the battery quickly, can be pro-arrhythmic and has a negative impact on the patients QOL.

Device programming to detect VAs is central to the delivery of appropriate and inappropriate therapies and should be based on a good understanding of the patient's arrhythmias, underlying pathophysiology and indication (primary/secondary) for ICD implant. Most devices allow for multiple zones of VA detection based on heart rate. Whereas a shock box programmed to detect and treat VF in a single zone works to reduce SCD, the use of VT zones allows for the use of ATP and SVT discriminators to avoid both

inappropriate and appropriate shocks. Careful device programming is key to achieve the primary aim of reduction of SCD while minimising the adverse effects of shock therapy.

1.2.3 ICD For Prevention of SCD – The Evidence Base

On a background of optimal medical therapy, RCTs have shown a risk reduction of sudden death following ICD implantation in patients with left ventricular dysfunction post MI to be approximately 25 - 50%. [1,24,25] These were primarily designed to test the efficacy of defibrillator therapy against pharmacological therapy alone for "protection" against SCD. Primary prevention trials investigating role of ICDs in potentially reducing the burden of SCD are summarised as follows:

MADIT I (Multicenter Automatic Defibrillator Implantation Trial I); 1996: was the first RCT designed to test if ICD therapy would result in improved survival in patients with CAD at high risk of SCD when compared to conventional medical therapy. 196 patients with ischaemic cardiomyopathy and a LVEF ≤ 35%, a documented episode of non-sustained ventricular tachycardia (NSVT) on holter monitoring and inducible VT by programmed stimulation (with failure of intravenous procainamide to prevent inducibility) were randomised to receive an ICD (n=95) versus conventional medical therapy (n=101). ICD therapy was associated with a 59% relative risk reduction of death after a mean follow-up of 27 months. This led to the approval of ICD for MADIT I indication 1996. [26]

MADIT II (Multicentre Automatic Defibrillator Implantation Trial II); 2002: following on from MADIT I results, this trial aimed to identify patients with ischaemic cardiomyopathy who are at high risk of SCD, before the actual manifestation of VAs. Patients with CAD with previous MI with LVEF ≤ 30% and on optimal medical therapy, were randomised to receive an ICD (n=742) or

no ICD (n=490). Results indicated a 31% relative risk reduction in mortality (SCD) in the ICD group over a 20-month follow-up. The FDA approved ICD prescription for MADIT II indication in 2002. Also, the American College of Cardiology and European Society of Cardiology rendered level IIA recommendation for prophylactic implantation of ICDs in MADIT II type patients. [26,27]

Trial	Year	Patients, (n)	LVEF (%)	Enrolment Criteria	Outcome
MADIT I	1996	196	≤35	Prior MI, LVEF≤35%, NSVT on Holter, inducible VT and failure of VT suppression with i.v. procainamide	54% reduction in total mortality with ICD therapy
MADIT II	2002	1232	≤30	Prior MI and LVEF≤30%	31% reduction in total mortality with ICD therapy
MUSTT	2000	1397	≤40	Prior MI and LVEF≤40%	31% reduction in total mortality with ICD therapy
SCD-HeFT	2006	1676	≤35	Ischaemic and non-ischaemic cardiomyopathy, LVEF≤35%, NYHA class I/II	23% reduction in mortality with ICD therapy
DEFINITE	2004	485	≤35	Non-ischaemic dilated cardiomyopathy, LVEF≤35%, NSVT or PVCs	35% reduction in arrhythmic mortality with ICD therapy
CABG-PATCH	1997	900	≤36	CAD, undergoing non-emergent CABG, LVEF≤36% and abnormal SAECG	No reduction in total mortality with ICD therapy
DINAMIT	2004	674	≤35	MI within past 40 days, LVEF≤35%, depressed HRV, average 24hr HR≥80bpm	50% reduction in arrhythmic deaths with ICD therapy
COMPANION	2004	1520	≤35	NYHAIII/IV, LVEF≤35%, QRS≥120ms, hospitalisation for CHF within 12 months	24% reduction in total mortality with CRT alone
MADIT- CRT	2009	1820	≤30	Ischaemic or non-ischaemic cardiomyopathy, LEVF≤30%, QRS≥130ms, NYHAI/II	34% relative risk reduction in risk of total morality

Table 1: Summary of primary prevention ICD clinical trials [19]

[Trial names, year in which trial carried out, number of patients recruited, their LVEF, enrolment criteria and trial outcomes have been shown. MADIT I: Multicenter Automatic Defibrillator Implantation Trial I, MADIT II: Multicentre Automatic Defibrillator Implantation Trial II, MUSTT: Multicentre Unsustained Tachycardia Trial, SCD-HeFT: Sudden Cardiac Death in Heart Failure Trial, DEFINITE: Defibrillators in Non-ischaemic Cardiomyopathy Treatment Evaluation, CABG-Patch: Coronary Artery Bypass Graft Patch, DINAMIT: Defibrillator in Acute MI Trial, COMPANION Trial: Comparison of Medical Therapy, Pacing, and Defibrillation in Heart Failure, MADIT-CRT: Cardiac-resynchronisation therapy for prevention of heart failure events]

MUSTT (Multicentre Unsustained Tachycardia Trial); 1999: tested the value of electrophysiologically guided antiarrhythmic drug therapy against no therapy in high risk CAD with poor LVEF ≤ 40% and NSVT. Risk assessment was carried out by testing inducibility of a sustained ventricular tachycardia. Patients with a history of MI, LVEF ≤ 40%, NSVT, and inducible VT despite antiarrhythmic drug use were randomly assigned to receive an ICD, electrophysiologically guided antiarrhythmic therapy, or no antiarrhythmic therapy. At 5 years, patients in the ICD group had a 31% absolute and 58% relative risk reduction. Electrophysiologically guided antiarrhythmic therapy did not reduce the rate of cardiac arrest, death from arrhythmia, or the overall mortality rate in comparison with no antiarrhythmic therapy. These results confirmed inducibility testing as the most accurate risk-stratifying marker and demonstrated no benefit in serial EP drug testing and highlighted the risks associated with stand alone antiarrhythmic drug treatment. [28]

SCD-HeFT (Sudden Cardiac Death in Heart Failure Trial); 2005: was designed to evaluate whether amiodarone or a conservatively programmed shock-only, single-lead ICD decreases the risk of death from any cause in a broad population of patients with mild-to-moderate heart failure. The trial enrolled 2521 patients with NYHA II/III chronic heart failure and LVEF ≤ 30% due to ischaemic or non-ischaemic etiology. Patients were randomised to receive

optimal medical therapy alone (n=847), optimal medical therapy along with amiodarone (n=845), or optimal medical therapy along with a ICD (n=829). Placebo and amiodarone were administered in a double-blind fashion. Results indicated a 23% reduction in mortality in the ICD group over a mean follow up of 3.8 years. The benefit of ICD was similar in both the ischaemic and non-ischaemic group. Optimal medical therapy and amiodarone lead to no survival benefit. [25]

DEFINITE (Defibrillators in Non-ischaemic Cardiomyopathy Treatment Evaluation); 2004: trial enrolled 458 patients with non-ischaemic dilated cardiomyopathy, LVEF ≤ 36%, and premature ventricular complexes or NSVT. Patients were randomised to receive standard medical therapy (n=229) or to receive standard medical therapy plus a single-chamber ICD (n=229). Results indicated a mortality rate of 14.1% in the standard medical therapy group and 7.9% in the ICD group at 2 years of follow up. The largest benefit was seen in NYHA class III patients. The results of this trial expanded the prescription of primary prevention ICDs to the non-ischaemic cardiomyopathy. [29]

CABG-Patch (Coronary Artery Bypass Graft Patch); 1997: trial investigated the benefit of ICDs in patients with CAD who underwent elective coronary artery bypass surgery and who also had a LVEF ≤ 36% and abnormal signal averaged electrocardiograms. 1055 patients were enrolled and randomly assigned to therapy with ICD (n=446) or to the control group (n=454). The primary end point of the study was overall mortality, and the two groups were compared in an intention-to-treat analysis. Contrary to other primary prevention trials, results indicated no survival benefit associated with ICD therapy. [30]

DINAMIT (Defibrillator in Acute MI Trial); 2004: examined the utility of early ICD implantation post MI in CAD patients with LVEF ≤ 35% and impaired cardiac

autonomic function (manifested as depressed heart-rate variability or an elevated average 24-hour heart rate on Holter monitoring). Patients were randomised to ICD therapy (n=332) or no ICD therapy (n=342), 6 - 40 days post MI. At 2.5- year follow-up, there was no survival benefit from early ICD implantation, which was associated with lower arrhythmic mortality rates but with an unexplained increase in non-arrhythmic death, in comparison with the outcomes after conventional therapy. The results of this trial lead to the recommendation that ICD implants should be delayed for at least 40 days following an acute myocardial infarct. [31]

COMPANION Trial (Comparison of Medical Therapy, Pacing, and Defibrillation in Heart Failure); 2004: is the only trial in which ICD therapy was evaluated in patients with advanced heart failure, NYHA class IV. The trial investigated the utility of prophylactic cardiac-resynchronisation therapy (with or without a defibrillator) in reduction of risk of death and hospitalisation among patients with advanced chronic heart failure and intra-ventricular conduction delay. 1520 patients who had advanced heart failure due to ischemic or non-ischaemic cardiomyopathies and a QRS interval of at least 120ms were randomly assigned in a 1:2:2 ratio to receive optimal medical therapy alone or in combination with cardiac-resynchronisation therapy with either a pacemaker or a pacemaker-defibrillator. The primary composite end point was the time to death from or hospitalisation for any cause. Results indicated that heart-failure hospitalisation and all-cause mortality rates were lower in both the CRT and CRT-D groups (24% versus 36%) than in the group that received no device therapy with a similar benefit across ischaemic and non-ischaemic cardiomyopathy patients. The results of this trial established a case for CRT-D

implants in patients with advanced heart failure and wide QRS interval with view to improve heart failure symptoms and reduce mortality. [32]

MADIT-CRT (Cardiac-resynchronisation therapy for prevention of heart failure events); 2009: was designed to evaluate the utility of CRT-D therapy in patients with mild heart failure. 1820 patients with ischaemic or non-ischaemic cardiomyopathy, LVEF ≤ 30%, QRS duration ≥ 130ms and NYHA class I/II were randomly assigned in a 3:2 ratio to receive CRT plus an ICD (n=1089) or an ICD alone (n=731). The primary end point was death from any cause or a nonfatal heart failure event. During a follow-up of 2.4yrs, a relative risk reduction of 34% was noted in the CRT-D group largely driven by a decrease in non-fatal heart failure events. Since CRT-D significantly reduced the incidence of the trial primary end point (without conferring a survival benefit from SCD prevention), it is now indicated in patients with NYHA class I/II. [33]

These trials provide conclusive evidence for mortality reduction from SCD and survival benefit provided by ICD therapy in ischaemic cardiomyopathy patients. Of note, different risk factors have been used to determine patient eligibility (and hence risk of SCD) for enrolment across trials. The unifying risk predictor appears to be a poor LVEF. A better understanding of various risk stratification markers used for primary prevention ICD implants and the evidence behind their utility in clinical practice is essential.

1.2.4 Current Risk Stratification for Primary Prevention ICDs

It is intuitive that the benefit derived from ICD therapy would be a balancing act between a patient receiving lifesaving appropriate therapy from the device or being lost to death in the absence of any device therapy. The key to achieving the former lies in timely and precise identification of patients with CAD who are likely to be at the highest risk of malignant VAs and hence SCD.

As evident from the various clinical trials discussed in the previous section, acute MI survivors are currently risk assessed and selected for primary prevention ICD implants largely on the basis of their LVEF. However, it is also apparent from these trials and more recent evidence that other variables in addition to LVEF, help refine risk of SCD.

These risk stratifying markers detect: (1) slow conduction (QRS duration, Signal averaged electrocardiogram – SAECG), (2) heterogeneities in ventricular repolarisation (QT interval, QT dispersion, T-wave alternans), (3) imbalance in autonomic tone (heart rate variability, heart rate turbulence, heart rate recovery after exercise, baroreceptor sensitivity), (4) extent of myocardial scar formation (LVEF, 6 minute walk), and (5) ventricular ectopy (long term ambulatory monitoring). [34] The current evidence for the use of these risk - stratifying markers is presented here.

Left ventricular ejection fraction (LVEF): is the most widely used measure of left ventricular systolic function. It is the single major parameter that has been most consistently studied in clinical trials and reported to reliably define risk for SCD in ischaemic cardiomyopathy. RCTs have demonstrated that LVEF is the strongest predictor of SCD in acute MI survivors. [25,26,28,29] The MADIT trial demonstrated a 50% reduction in mortality with ICDs compared with medical therapy alone. [26] Subsequent analysis of the MADIT data demonstrated that this benefit was greatest in patients with LVEF ≤ 26%. [35] These results were reinforced in MADIT II which demonstrated a 31% risk reduction of death in patients (with LVEF ≤ 30% and prior MI) who were randomised to the ICD arm. [26] Likewise, it was noted in the MUSTT trial that the frequency of arrhythmic deaths was higher in patients with LVEF ≤ 30%. [28]

Similar reduction in mortality with ICD therapy in patients with poor LVEF were seen in the SCDHeFT trial. [25]

LVEF is commonly measured by 2-dimensional echocardiography and has several advantages. It is readily available and routinely performed in patients post MI. The accuracy of LVEF is in excess of ± 10% for both visual estimation and calculation by Simpson's rule with echocardiography. Current international guidelines for prophylactic ICD implants are predominantly based on LVEF.

QRS duration: on a 12 lead electrocardiogram (ECG) quantifies the duration of ventricular depolarisation and is a reflection of intra-ventricular or inter-ventricular conduction delay or block. It is highly reproducible with a coefficient of variation of < 5%. [36] QRS prolongation has been shown to be associated with worse outcome in patients with poor LVEF due to CAD in observational studies. [37] QRS prolongation could be a surrogate marker for more advanced myocardial disease, but it may also contribute directly to increased mortality, because dys-synchronous ventricular activation may cause depression of cardiac function. [34] The CASS (Coronary Artery Surgery Study) registry showed that patients with left bundle branch block (LBBB) had more extensive CAD and worse LVEF than patients with a normal QRS. Moreover, LBBB was a strong predictor of arrhythmic mortality independent of degree of heart failure and extent of CAD. [38] The presence of fragmented QRS (fQRS) has been shown to be associated with myocardial scar in CAD patients and evidence suggests that it is a significant predictor of mortality and SCD in CAD. [39]

The utility of QRS prolongation as a predictor of SCD has been tested in subgroup analysis of various RCTs. An independent analysis of MADIT II data revealed that a prolonged QRS was significantly associated with survival

benefit from ICD therapy. [40] Prolonged QRS (>120ms) was also shown to correlate with appropriate ICD therapy and a higher hazard ratio of SCD (ICD therapy). [25] MUSTT results demonstrated that LBBB and non-specific intraventricular conduction delay (IVCD) were associated with increased total mortality risk, although there was no significant link between LBBB and inducible monomorphic VT. [28]

The simplicity, ease of measurement, cost-effectiveness and easy reproducibility on each follow up visit make QRS duration an attractive candidate for further investigation. It is not currently used as an independent parameter for risk stratification of SCD in ischaemic cardiomyopathy.

QT interval and QT dispersion: QT interval represents the time required for both ventricular depolarisation and repolarisation to occur and hence is a reflection of summed ventricular action potential duration. It shortens with increasing heart rate and is commonly corrected (QTc) by using the Bazett's formula. Although, QT-interval measurements have been shown to be highly reproducible, [41] the need for rate correction with suboptimal formulas limits the comparability of QT data in populations. [34]

Observational studies have shown association of QT prolongation with mortality in patients with moderate and severe left ventricular dysfunction. [42] However, this association was not reproducible in other studies. [43,44] Some population studies have demonstrated QT prolongation to independently effect the prognosis of cardiovascular mortality, [45] but its clinical utility (in the absence of congenital long QT syndrome) is at best limited to a risk association with increased mortality in CAD. [46]

QT dispersion represents the difference between maximum and minimum QT interval on a 12-lead ECG. It is thought to measure cardiac

electrical heterogeneity and hence of value in arrhythmia risk prediction. It has been associated with increased mortality in some observational studies. [42] Yet, others have found no association between this parameter and cardiovascular outcome. [43,47]

The utility of QT interval and QT dispersion in ischaemic cardiomyopathy has not been tested in any large RCT. They are not used as independent risk stratifiers in this context. The inter- and intra-observer variability reduce the reproducibility of these parameters, which further challenges their utility for risk stratification.

Holter Analysis: Ventricular ectopy and NSVT – ventricular premature beats and NSVT have been shown to have prognostic importance in patients with CAD especially in the presence of left ventricular impairment. An increased relative risk of SCD in patients post MI with ≥ 10 VPB/hour and NSVT has been demonstrated. [48-51] This observation was reproducible in the modern era of interventional cardiology and early re-perfusion. [49,52,53] NSVT was found to be an independent predictor of SCD post MI. [53] The European MI Amiodarone Trial (EMIAT) investigators showed a higher mortality in patients with frequent or complex arrhythmias on AECG than in those without (20% versus 10%) on the background of poor LVEF ≤ 40% post MI. [54]

In combination with an impaired left ventricular function, ventricular ectopy and NSVT are very strong risk factors for mortality in CAD. Unfortunately, the sole use of conventional 24-h Holter monitoring for the prediction of VAs is significantly limited by its low sensitivity and specificity.

Heart rate variability (HRV) – provides a surrogate for the autonomic effects in the ventricle that are postulated to be important in the pathogenesis of VAs. It

has been proposed that an analysis of HRV, particularly its parasympathetic effects on the sinus node, can potentially predict mortality. [34]

HRV reflects changes in cardiac autonomic tone that occur post MI. It tends to decrease early after an infarct and a steady increase towards normality is observed within 6 – 12 weeks. [55] The Multicentre Postinfarction Study (MPS) showed that HRV was a significant predictor of mortality in acute MI patients, and remained so even after adjusting for LVEF. [56] The ATRAMI (Autonomic Tone and Reflexes after MI) trial provided clinical evidence that after MI, patients with low HRV had a relative mortality risk of 3.2 having accounted for a poor LVEF. However, it also demonstrated that other markers of autonomic dysfunction were superior to HRV in prediction of mortality in context of a poor LVEF (≤ 35%). [57] In the DINAMIT and ALIVE trials, patients with low HRV had a higher non-arrhythmic mortality, which was believed to be a reflection of advanced haemodynamic compromise. [31,58]

HRV can be automatically recorded using the standard holter. Overall low HRV has been shown to be a predictor of overall mortality rather than being specific for arrhythmic deaths. This alongwith its futility in patients with atrial fibrillation and its variability with external factors like age, gender and functional status of sinus node, limits its use as an independent risk predictor in ischaemic cardiomyopathy.

Heart rate turbulence (HRT): is a marker of baroreceptor sensitivity that quantifies mainly the reflex vagal activity. It is a measure of fluctuations in sinus rhythm cycle length following a single premature ventricular ectopic. In normal healthy individuals, a premature ventricular ectopic is followed by a biphasic response (acceleration followed by deceleration) of sinus rhythm. This pattern is absent in high-risk patients. HRT has been shown to be a powerful

independent predictor of risk following a MI. The REFINE (Non Invasive Risk Assessment Early after a MI) study results concluded that impaired HRT in patients with LVEF ≤ 50%, beyond 8 weeks after a MI could reliably identify patients at a high risk of arrhythmic death. [59] HRT in combination with deceleration capacity was shown to identify high-risk individuals amongst post MI patients with LVEF ≤ 30%. [60] Moreover, a relative risk of ~ 4 for fatal and non-fatal cardiac arrest post MI was attributable to a low HRT in an ATRAMI substudy. [61]

HRT has primarily been examined in patients with ischaemic etiologies. It can be automatically recorded with a standard holter. However, it cannot be reliably assessed in patients with atrial fibrillation and an optimal time post MI to perform the test has not been established. Given the results so far and the ease with which it can be performed, it is a potentially attractive risk stratification tool.

Signal Averaged ECG (SAECG): calculates an average of consecutive QRS complexes (usually around 300) to produce a filtered QRS complex that provides useful information on the presence of ventricular late potentials (VLPs). VLPs represent slowed conduction through a diseased myocardium, which may serve as a substrate for subsequent VAs, and have been documented in 25-50% of patients soon after MI. [62] Various studies have looked into the prognostic value of SAECG in predicting mortality post MI. SAECG has a sensitivity of 15% to 75% (over a follow up period of 6 – 24 months) to predict arrhythmic events with a negative predictive value of >90% and hence its utility in identifying very low risk patients. [62,63]

The MUSTT investigators found that a filtered QRS >114ms was associated with a 28% risk of arrhythmic events during a 5-year follow-up

period compared with a 17% risk of events for those with shorter filtered QRS durations. [64] The CABG-Patch trial used SAECG as an enrolment criteria in addition to poor LV function but did not find it useful in defining arrhythmic risk.[30]

SAECG is easy and quick to perform and can be utilised in patients with atrial fibrillation. However, it has a very low positive predictive value and hence limiting its utility in identification of high-risk patients. Also, it is better at predicting ventricular tachycardia rather than ventricular fibrillation and a standardised measurement in the presence of bundle branch block is not available. It is currently not used as a sole risk stratification marker and is recommended as a class IIB risk stratification tool in post MI patients. [65]

T wave alternans (TWA): is a heart rate dependent measurement of beat-to - beat changes in the amplitude of the T-wave on an ECG. It is thought to reflect dispersion of ventricular repolarisation and has been shown to be associated with fatal VAs. [66,67] TWA can be assessed during exercise (microvolt T-wave alternans, MTWA) or during ambulatory Holter-based recordings (modified moving average analysis). MTWA has been shown to be a strong predictor of fatal arrhythmias and SCD post MI, with and without left ventricular impairment. [66,67] The ABCD (Alternans before Cardioverter Defibrillator) trial was a prospective trial in ischaemic cardiomyopathy patients (LVEF ≤ 40%) and demonstrated that risk stratification using MTWA versus invasive electrophysiology studies are comparable at 1 year and complementary when used in combination. The positive and negative predictive values of MTWA at 1 year were 9% and 95% respectively. [68] Another similar trial, the MASTER (Microvolt T wave Alternans Testing for Risk Stratification of Post-Myocardial Infarction Patients) trial in ischaemic cardiomyopathy patients showed that

MTWA testing did not predict arrhythmic events. However, MTWA non-negative patients (positive and indeterminate) had significantly higher mortality than MTWA negative patients. [69]

TWA currently has a class IIa indication for risk stratification of primary prevention ICDs in ischaemic cardiomyopathy. Its high negative predictive value makes it valuable for identification of low risk individuals. However, a significant proportion of tests are indeterminate due to inability to achieve a target heart rate with regular R-R intervals; secondary to atrial fibrillation, frequent ectopic activity or failure to reach a target heart rate. Its value may be enhanced in combination with other risk predictors.

Baroreceptor sensitivity (BRS): is used for assessment of autonomic control of the cardiovascular system. It refers to the adaptation of R-R intervals to changes in blood pressure. [70] BRS is most commonly measured in clinical practice during a brief period of controlled blood pressure change while administering an intravenous bolus of phenylephrine.

BRS has been shown to be strongly associated with a risk of fatal VA. In a prospective study of 78 post MI patients, BRS was found to be significantly lower in the deceased patients (including SCD) than in the survivors. [71] These results were found in other studies as well. [72,73] The multicenter ATRAMI study also helped establish BRS as a risk stratification marker in ischaemic cardiomyopathy. [57] Depressed HRV or BRS were shown to have a significant risk of cardiovascular mortality and this risk was further increased if both parameters were depressed. Also, further analyses showed that in conjunction with a poor LV function (LVEF<35%), a depressed BRS conferred a significantly worse two-year survival than a preserved BRS in these patients.[57]

BRS may be useful in risk stratification for SCD in ischaemic cardiomyopathy, but it is not yet established as an independent marker.

It is evident from the above discussion that left ventricular dysfunction remains the key investigation in determining which patients should be offered an ICD. A number of non-invasive investigations have been shown to help in risk stratification. However, these have not found a place in the current international guidelines.

With an increasing number of patients surviving acute MIs, the number of ICD implants has substantially increased in this group of patients at a high risk of VAs. [2,3,5] Subjecting an increasingly large population to an invasive ICD implant requires more thought as it has clinical implications for the patients and financial implications for the healthcare providers. To be able to use resources efficiently for the greatest clinical benefit, it is important to understand not just the clinical benefits of an ICD implant but the associated clinical procedural risks and financial implications.

Without doubt, the ICD has proven its utility in reducing mortality in a very high-risk population. But do ICD implants come with their own risk? Or is there a dark side to life with an ICD implant? Or perhaps, do we need to and can we, better identify patients who would derive maximum benefit from an ICD?

1.2.5 Problems with ICD Implantation

Although ICDs are the most effective means of preventing SCD, they remain expensive and are associated with significant co-morbidity. The major challenges that health care providers and the patient population are faced with are:

Procedural risks: ICD implantation is an invasive procedure that comes with its attendant risks. Recorded risks/complications include pain, bruising, radiation exposure, haematomas, infection, pneumothorax, lead displacement and cardiac tamponade. Procedural complications associated with ICD implantation occur in 11% of patients and around 1% patients develop a device infection requiring risky lead extraction. [74]

Cost-Effectiveness: NICE has estimated the average cost of an ICD implant in the UK at approximately £9692 excluding hospitalisation and follow up costs. The initial treatment of patients at increased risk of SCD with ICDs in combination with optimal medical therapy had an incremental cost-effectiveness ratio (ICER) of £19,479 per QALY gained compared to optimal medical therapy alone. The ICERs in other groups including chronic heart failure due to ischaemic and non-ischaemic causes ranged between £14,231 and £29,756 per QALY gained. [2]

Buxton et al conducted a cost-effectiveness model for primary prevention ICD implants in the UK. Mean discounted gain from ICD implant (at a high financial cost) over a 20yr horizon was 1.24 years or 0.93 QALYs. They concluded that ICDs, as currently applied in the UK, are not cost-effective by conventional standards. [75] Similar concerns over cost-effectiveness of ICDs have been raised by other investigators as well.[74,76] It has been suggested that the best utilisation of finances for maximum clinical benefit can be achieved by better risk stratification in order to implant ICDs in high risk patients without associated co-morbidities that limit life expectancy to 10 years and by reduction of follow up costs. [77]

Psychological Impact and Quality of Life (QoL): While many patients feel secure and comforted by having the ICD implant as a “safety-net”, others

commonly have ICD related fears including impending shocks, public embarrassment and death. Shocks (both appropriate and inappropriate) have an adverse psychological impact that has been well documented in the QoL analysis from various trials including AVID and DEFINITE. [76]

QoL studies have demonstrated that patient concerns about the ICD are an important indicator of psychosocial adjustment. Although patients who had experienced a shock scored higher on ICD concerns, ICD concerns was the only independent determinant of anxiety and depressive symptoms adjusting for shocks and all other factors. [78] Anxiety levels and depression have been found to be high post ICD implant and shock therapy and decrease over time from the primary event. [79]

Inappropriate device therapy: Out of all primary prevention ICD implants only 20-30% patients ever get treatment from it and not all device therapies are life saving. [80] A vast majority of patients experience inappropriate shocks from the ICD, their incidence varying between 15 – 35%. [81] Inappropriate ICD shocks have a negative impact on the patients' QoL, increase health care resource utilisation and can lead to other adverse sequelae. Not only are these painful and psychologically damaging but have been found to be associated with a worse prognosis with a hazard ratio for death of 1.98. [82]

Supraventricular arrhythmias with a rapid ventricular response are the commonest cause of inappropriate device therapy. Statistically significant predictors of inappropriate ICD interventions in the MADIT II study included atrial fibrillation (AF), smoking, and diastolic blood pressure > 80 mm Hg. Other factors contributing to inappropriate therapies include abnormal ICD sensing (R/T oversensing, far field electrograms, diaphragmatic potentials), ICD failure and electromechanical interference. [83] The potential of ICDs being pro-

arrhythmic has been reported. [84] Device pro-arrhythmia can be attributed to local effects from leads, direct and indirect effects of ventricular pacing, and problems associated with improper device function. While the concept of local irritability is supported by a relatively higher incidence of VAs in the first week after transvenous and epicardial ICD implantation, local fibrosis can occur at any time after lead implantation and is more difficult to demonstrate. Change in electrical activation associated with ventricular pacing is another plausible mechanism for pro-arrhythmicity of ICDs. [85]

Hardware malfunction: despite advances in ICD system technology and manufacturing, structural failure of an implanted device is not unheard of. The estimated survival rate of ICD leads was noted to be 85% at 5 years and 60% at 8 years after implantation. [86] Advisories on potential for ICD malfunction were issued by all major device companies in the year 2005. This was followed soon after by the recall of the Sprint Fidelis lead due to lead fractures resulting in an increased risk of inappropriate shocks. [87,88] While the impact of this on patients is intuitive, it was particularly challenging for the clinicians as well who were left to manage these patients based on individual experience.

1.2.6 Need for Improved Risk Stratification of SCD

The vast majority of patients who qualify for an ICD implant represent a very high-risk group, with multiple co-morbidities at an advanced age. Numerous clinical features have been shown to be predictive of or associated with a higher risk of VAs. And yet, the most clinically useful and accepted in international guidelines is only one – LVEF. Despite the proven survival benefit of ICD prescription, only a minority of patients (<1/3rd) [80] ever use it; while still being subject to the challenges associated with ICD implant. In order to

improve the risk stratification process for ICD implants, the current practice trends and guidelines defining ICD prescription need to be examined.

Limitations of LVEF as risk stratification marker: Impaired LVEF has been shown to be a strong predictor of SCD and currently forms the basis of primary prevention ICD implants in ischaemic cardiomyopathy. However, only 20-30% patients in primary prevention ICD trials ever receive appropriate device therapy; [80] in contrast to 2/3rd of the victims of SCD in population based studies with a normal or mildly impaired LVEF. [89] The vast majority of MI survivors have a preserved or mildly reduced LVEF and they still go on to have a large number of SCD events despite the presumed low risk. In the Maastricht Circulatory Arrest Registry, 51% of victims of sudden circulatory arrest with echocardiography during the study period had an LVEF >40%. [90] The specificity of LVEF as a marker of SCD is questioned by these observations. The sensitivity and specificity of LVEF for major arrhythmic events post-MI was found to be 59% and 78% respectively in a meta-analyses of 20 studies. [91]

In the high-risk risk group with LVEF ≤ 35%, only a small proportion benefit from ICD implant. Primary prevention ICD trials have demonstrated that only 20-30% of patients, under current prescription, receive an appropriate ICD therapy. [80] The SCD rate in MADIT II was 10% in the conventional arm and 3.8% in the ICD arm. Although, this was higher in the conventional arm, the vast majority of these “high-risk” patients would not benefit from ICD therapy by virtue of not having had a cardiac arrest. [26] Similarly, the rate of fatal VAs was only 8% in the CARISMA (Cardiac Arrhythmias and Risk Stratification after Myocardial Infarction) and 5% in MUSTT trial over a follow-up period of two years. [92,28] Thus the risk associated

with a poor LVEF is not uniform across this “high-risk” subgroup. Moreover, a reduced LVEF is a marker for both SCD and total mortality.

While various guidelines specify LVEF criteria for device implant, the technique by which this should be measured is not specified. Echocardiography is the most commonly used modality in clinical practice. However, the reliability of echocardiography and the associated inter and intra-observer variability has been questioned. It has been shown that CMR has greater reproducibility than echocardiography in assessment of LVEF. Also, using CMR as the reference standard, echocardiography was found to systematically overestimate EF by an average of 3 percentage points (37.5% versus 34.4%). And hence, using strict LVEF criteria, CMR would have resulted in almost 21% of patients being reclassified regarding ICD eligibility. [93] The prognostic ability of LVEF is heavily influenced by the measurement modality being used. Also, a single time point measurement might not suffice as left ventricular function is subject to change with evolution of the underlying disease process and also with various pharmacological and invasive interventions.

Limitations of Clinical Trial Design: the dominant parameter governing entry into most primary prevention ICD trials has been LVEF. However, this entry requirement has not been stratified with a different threshold being used across clinical trials. The entry threshold varied between a LVEF ≤ 30% to ≤ 40%. The LVEF of patients actually enrolled into the trial and those deriving benefit from device therapy was not always in line with the entry threshold. [19] The entry threshold for SCDHefT was ≤ 35%, while the median LVEF amongst enrolled patients was 25% with an interquartile range of 20% - 30%. Moreover, subgroup analysis of patients with LVEF ≥ 30% (constituting 17% of all recruits)

was indicative of no ICD benefit. [25] Also, to note there is no time specified to obtain the LVEF, which determines eligibility for an ICD. This could be measured early on (≥4 weeks) post MI or several years after the index event.

The clinical implications of LVEF are not limited to manifestation of SCD. Rather, both a poor LVEF and SCD are inseparable from heart failure. The presence or absence of heart failure has a significant bearing on the benefits derived from the ICD and its associated risks. Heart failure has been used as an entry criterion in some but not all clinical trials and there is no apparent uniformity in its selection. A high prevalence of heart failure was found in study patients in clinical trials which used it as an entry criteria (DEFINITE and SCDHeFT) [25,29] and in those who did not (MADIT II). [26] Subgroup analysis of the MADIT population also suggested that an EF< 25% and/or a history of heart failure were better predictors of ICD use and mortality during follow-up than EF <26% without heart failure. [19,26] Interactions between LVEF and heart failure should be taken into account when risk stratifying patients.

The study populations in the vast majority of ICD trials may not be a true reflection of the general population. The elderly population (age ≥ 80yrs) was largely excluded from most trials. Varying benefit with age has been in subgroup analysis of various trials. Patients over 70yrs of age had a similar benefit from ICD implant as the younger population in MADIT II. [26] This was not true in the SCDHeFT population. [25] Patients with renal dysfunction on haemodialysis have been excluded from most trials as well. The high prevalence of CAD in these patients increases their risk of fatal arrhythmias. However, this might be largely confounded by the likely increased incidence of total mortality in this population given their multiple co-morbidities. In addition, subgroup analysis from MADIT II showed that patients with advanced renal

disease did not demonstrate a mortality benefit from ICD implantation. [26] Female population is not very well represented in the ICD trials. This might be a reflection of lower incidence of CAD in this gender. However, a clear difference in ICD benefit between men and women has been seen in some ICD trials. [32] This needs to be explored further.

Critical analysis of the existing evidence does suggest that ICDs have a clear survival benefit in high-risk patients with severely impaired left ventricular function post MI. However, the current risk stratification is by no means perfect. Improved risk stratification is required so that patients receiving ICDs will derive maximum benefit, while those not expected to achieve much benefit are excluded from consideration of device therapy. ICDs are fraught with morbidity and mortality. The general population needs to be protected from these risks while making sure that those at highest risks are not denied of survival benefit. While deriving maximum clinical benefit, it is also crucial to make ICD therapy cost-effective.

The dominant risk stratification marker in primary prevention ICD trials has been LVEF. Alongwith other limitations discussed before, one of the conceptual flaws appears to be the use of a single time point measurements determining ICD prescription. The static nature of these designs does not take into account the dynamic pathophysiology of ischaemic heart disease that encompasses changes over time and is likely to influence the presence and stability of current risk markers. [94]

The concept of cardiovascular ageing and CAD being caused by premature biological ageing has emerged in recent years. This is in addition to and distinct from chronological ageing. On the background of a similar risk factor profile, an individual with accelerated biological ageing is more likely to

develop CAD and/or manifest its complications at a younger age when compared to someone who ages at a normal rate. An ideal risk marker would be able to capture this accelerated biological ageing early on and distinctly from chronological ageing and help predict the occurrence of cardiovascular events and SCD.

Telomere biology has recently been shown to capture cardiovascular ageing especially in the realm of CAD. Recent research has identified leukocyte telomere biology as a potentially important marker for a range of diseases and disease potential. [95-103] Telomere biology is a potential robust biomarker for cardiovascular events in CAD as it changes progressively with chronological age, has inherent variation amongst individuals, quantifies accelerated biological ageing by reflecting the replicative potential of the cell and also by indicating the cumulative lifelong burden of oxidative stress.

Before moving on to the telomere structure and their role in premature biological ageing, it is important to understand the difference between chronological and biological ageing.

1.3. The Biology of Ageing

1.3.1 Chronological and Biological Ageing

Ageing is the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease or death. Ageing per se is not a disease, but a natural process that can be distinguished from diseases by at least four criteria. [104,105] Unlike any disease, age related changes (1) occur in every animal that reaches a fixed size in adulthood (2) take place in virtually all species (3) occur in all members of a species only after the age of reproductive success (4) occur in animals removed from the wild and protected by humans.

An individual's age, as we commonly see it, is the chronological age, which is an extrinsic variable measured in calendar days/months/years. It is governed by a fixed, extrinsic timeline that progresses at the same rate in all individuals of every species. On the other hand, biological ageing, has a great variation amongst individuals (even of the same species), is governed by intrinsic variables and cannot be determined solely by fixed calendar times. The rate of biological ageing can be the same as the chronological ageing, slower or faster than chronological ageing. An individual who has a faster biological ageing rate than chronological ageing rate will have "premature biological ageing" as a result. This premature biological ageing, even in the absence of an additional disease process, will lead to accelerated cellular senescence and a shorter finite life span. Of the various stochastic and developmental-genetic theories of ageing, cellular senescence provides the most definitive mechanism, postulating a link between limited replicative capacity at a cellular level and biological ageing.

1.3.2 Limited Replicative Capacity

In the early 1920's, immortality of explanted cells was claimed on the basis of experiments carried out on chicken heart fibroblasts. It was suggested, that the only reason that these cells are unable to replicate continuously was that researchers were unsure as to how to cultivate them. [106] This observation was challenged in the 1960's by Hayflick, who demonstrated that normal human fibroblasts in culture had a finite replicative capacity and this was linked to cellular ageing and senescence.

Hayflick and Moorhead mixed equal number of normal human male fibroblasts that had gone through numerous divisions (cells at fortieth population doublings) with female fibroblasts that had undergone very few cell

divisions (cells at the tenth population doubling). Unmixed cell populations served as controls. When the male 'control' culture stopped dividing, the mixed culture was examined and only female cells were found. This experiment showed that the older cells "remembered" that they were old even in a mixed culture. And the concept of the Hayflick limit i.e. the number of divisions a given cell type is capable of, is now well accepted. [107]

Despite the landmark findings in cellular senescence, Hayflick's experiment did not explain what controls the number of divisions a cell is capable of or how the "older" cells knew they were older.

1.3.3 DNA Replication

DNA synthesis in eukaryotic cells is mediated by the enzyme DNA polymerase. Deoxyribonucleotides are added to the 3' hydroxyl terminus of DNA by the polymerase enzyme. The new DNA is synthesised as the parental DNA unwinds at the replication fork. Both strands of parental DNA act as a template. The overall direction of synthesis is 5' → 3' for one strand and 3' → 5' for the other strand. As DNA polymerase can only synthesise in the 5' → 3' direction, there is a "leading" and a "lagging" strand. The discontinuous assembly of the lagging strand enables 5' → 3' polymerisation at the nucleotide level to give overall growth in the 3' → 5' direction. DNA polymerase uses short primers to initiate DNA synthesis. These are removed once the synthesis is complete and the resultant gaps filled by ligation mediated by DNA ligase.

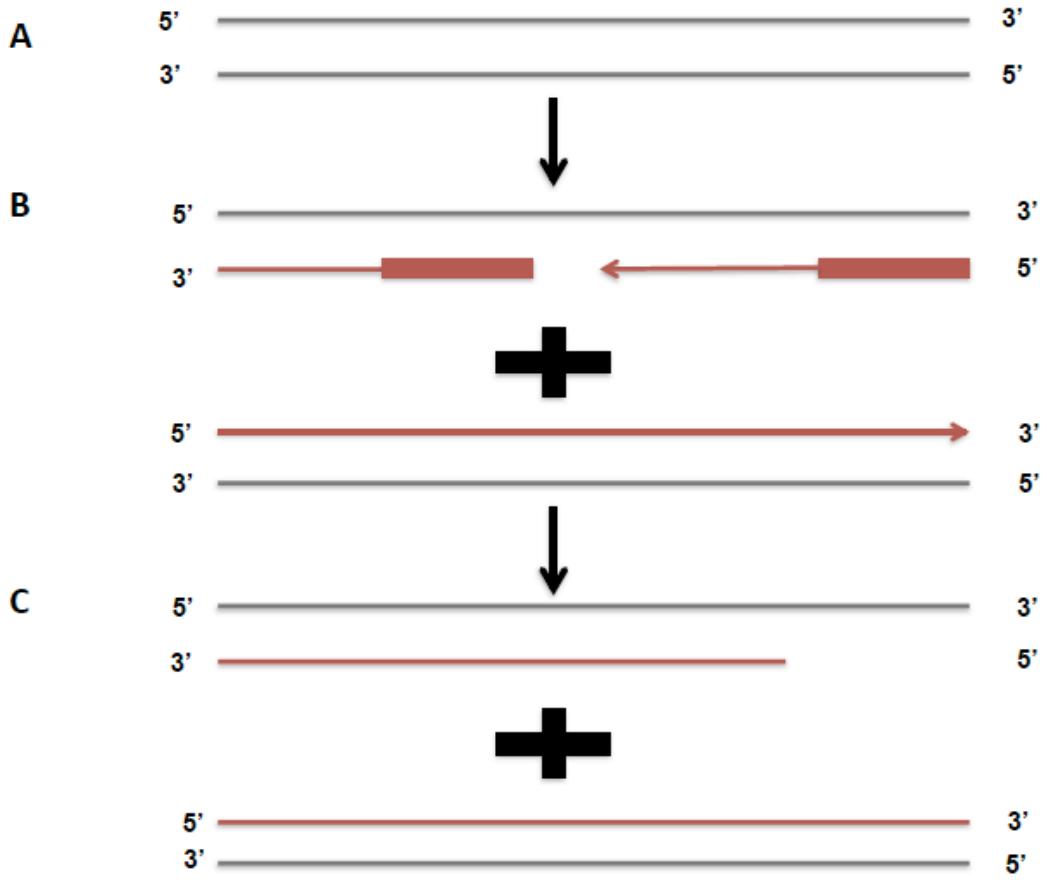

Figure 2: The End Replication Problem

[A. Parental DNA. B. Bottom strand synthesised by leading strand synthesis (5'→3') to the last nucleotide. Top strand synthesised by discontinuous lagging strand synthesis primed by RNA primers. C. RNA primers removed and filed in leaving a 5' gap].

Olovnikov pointed out the shortcoming of this mechanism of DNA synthesis. He noted that the DNA ligase can only join two strands together; at the every end of a linear DNA molecule there will be only one strand and so the newly synthesized DNA will be shorter than the template by the length of the primer. This is known as the "end replication problem" and forms the basis of the cellular mitotic clock. The relationship between this mitotic clock, cellular

senescence and biological ageing is explained by the presence of telomeres.[108]

1.4 Telomeres

1.4.1 Telomeres – Historical Perspective

Telomeres are the terminal sequences of linear eukaryotic chromosomes that "cap" the chromosomal ends and protect them from erosion. The word telomere originates from Greek *telos* meaning "end" and *meros* meaning "part". They were first identified in the work carried out by Herman Muller and Barbara McClintock in the 1930's and 1940's. As Muller noted from his experiments:

"...the terminal gene must have a special function, that of sealing the end of the chromosome, so to speak, and that for some reason a chromosome cannot persist indefinitely without having its ends thus sealed."

At that time their function was not very clear but there was evidence that telomeres prevented the ends of chromosomes from fusing to each other.[109,110] Muller noted that terminal deletions and inversions were rare post X-irradiation of chromosome ends. This level of chromosomal stability could only be possible with the help of special protective terminal ends. McClintock studied the broken chromosome ends of Zea Mays (maize/corn) and found that broken chromosome ends were reactive and fused with other broken ends. This was in contrast to normal, natural chromosome ends, which tend to be stable. Also, broken chromosome ends underwent degradation in the cell, which was subsequently harmful for the chromosome involved. And hence concluded that each chromosome end had a unique structure or sequence that identified it as natural and distinguished it from broken DNA. [110] In 1961, Hayflick's experiments on fibroblasts provided evidence that primary cells could only undergo a limited number of cell divisions and are not immortal. This

phenomenon, commonly known as Hayflick's limit, [111] confirmed the presence of a cellular counting mechanism. This cell cycle arrest described by Hayflick was then linked to the terminal ends of chromosomes by Olovnikov. [108]

The first telomeric sequence was discovered by Blackburn in 1978 in telomeres of ciliated protozoan, Tetrahymena thermophile, consisting of a simple sequence of hexameric repeats of the nucleotides TTGGGG. [112] The human telomeric sequence was discovered 10 years later by Moyzis and consists of a highly conserved hexameric TTAGGG repeats. [113] Once this sequence was known, the length of human telomeres could be measured. The possibility of telomeric shortening was described in 1986 when it was shown that telomere lengths are not same in all tissues. [114] Telomeric shortening was demonstrated in dividing fibroblasts in culture. [115] This observation in combination with other experiments, provided strong evidence for telomeric shortening limiting cell proliferation in culture. [115-117]

The next major landmark in telomere biology was discovery of the enzyme telomerase by Greider and Blackburn in 1985. [118] This was the potential solution to short telomeres limiting the rate of cell growth. The unique characteristic of telomeres in somatic cells is their age related attrition and their positive association with replicative capacity of cells in cell culture. The enzyme telomerase is the compensatory mechanism that overcomes this limitation by (by-passing the end-replication problem encountered by DNA polymerase) elongating telomeres. The structure of the human telomere and enzyme telomerase is described next.

1.4.2 Human Telomere and Telomerase

Telomere – structure and function: Telomeres are special functional complexes at the ends of eukaryotic chromosomes which protect the dsDNA ends and are

involved in maintain genomic stability and in regulation of cellular lifespan. [119] They consist of tandem repeats of TTAGGG sequence spanning several kilobases. The number of terminal hexameric repeats is variable and is shorter in somatic tissues (~ 10kb) than in germ cells (~15kb). The very distal ends of telomere comprises of a single stranded G-rich overhang, which is always orientated 5' – 3' and can be up-to 200 nucleotides long. [120] Adjacent to this is a complex region of segmentally duplicated DNA tracts called the subtelomeric repeat DNA. Human subtelomeric segmental duplications (subtelomeric repeats) comprise approximately 25% of the most distal 500kb and 80% of the most distal 100kb in human DNA. The overall size, sequence content, and organization of subtelomeric segmental duplications relative to the terminal $(TTAGGG)_n$ repeat tracts and to subtelomeric 1-copy DNA are different for each subtelomere. [121]

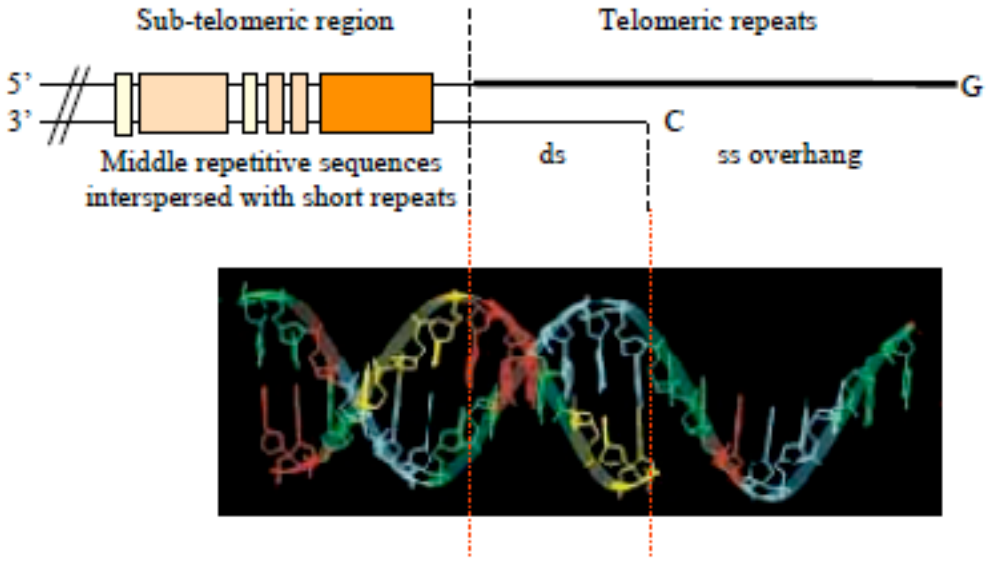

Figure 3: Human telomere structure

[Schematic representation of human telomere (upper) showing telomeric repeats and sub-telomeric region and the simplified 3D structure (lower)]

Telomeres protect the chromosomal ends from degradation and unwanted fusion events. It provides a means to replicate the chromosome completely by telomerase dependent replication or through recombination. Telomeres also serve to position the chromosome within the nucleus and are involved in the selective silencing of proximal subtelomeric genes. [105,121] Telomeres undergo attrition with each cell division and their length thus reflects the replicative history of cells and hence serving as a mitotic clock at least at the cellular level.

Telomeric complex: The 3' protruding G-rich strand can form complex structures of telomeres, the G-quadruplexes. These assume different conformations which can come in the way of DNA replication and telomere elongation by telomerase. [123] In the non-mitotic phase of the cell cycle this G-rich strand is shielded by the telomere shelterin complex in which the telomere binds internally to form the D and T-loops. The single stranded G-rich overhang can invade the pre-ceding double stranded region to form a telomere loop (t-loop). This can lead to invasion and displacement of upstream 3' telomeric DNA creating a displacement loop (D-loop) via base pairing which would contain three strands – two G-rich and one C-rich strand. A section of the terminal duplex may also invade resulting in a D-loop consisting of four strands – two G-rich and two C-rich. The D-loop is though to be essential for the stabilisation of the larger T-loops. [124] The formation of these loops depends on telomere binding proteins, especially TRF2. Increased amounts of TRF2 lead to more efficient G-rich overhang and hence T-loop formation which then protects the shortest telomeres in the cell and delays senescence. [125]

The shelterin complex is a group of protective telomere binding proteins and determines the protective function or "capping" function of telomeres.

These include telomere repeat binding factor 1(TRF1), TRF2, repressor/activator protein 1 (RAP1), TRF1-interacting nuclear protein 2 (TIN2), TIN2-interacting protein 1 (TPP1) and protection of telomeres 1 (POT1). In human cells, the best known telomeric proteins are TRF1 and TRF2 and both are negative regulators of telomeric length. Telomeres lacking TRF2 have been shown to signal p53 and ATM dependent apoptosis, [126] while overexpression of a dominant negative form of TRF2 induced end-to-end chromosome fusions and senescence. [127]

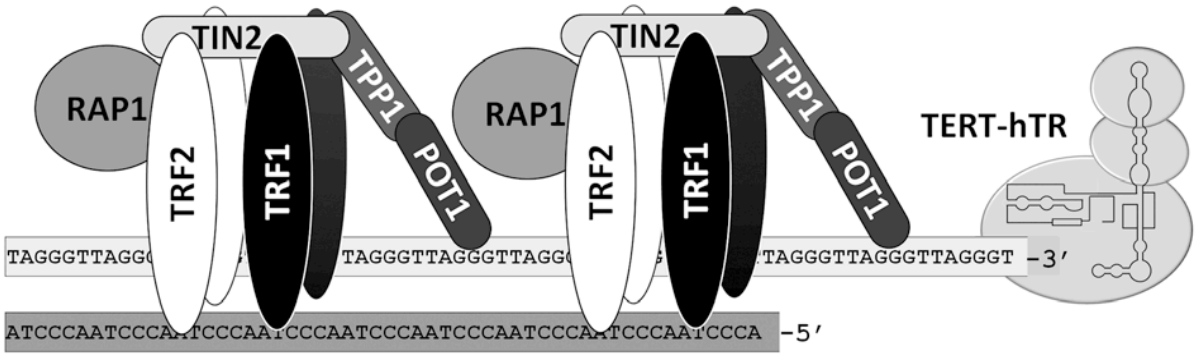

Figure 4: The Shelterin complex

[Schematic representation of telomeric binding proteins. RAP1: Repressor/Activator Protein 1, TRF1: Telomere Repeat binding Factor 1, TRF2: Telomere Repeat binding Factor 2, TIN2: TRF 1 Interacting Nuclear factor 2, POT1: Protector of Telomeres 1, TPP1: PIP1-POT1-TIN2 organizing protein]

(Taken from Int J Oncol 2012 Nov;41(5):1561-9) [128]

TRF1 and TRF2 are also involved in the ATM and ATR pathways, which are sensors of DNA damage. They can induce cell cycle arrest via kinase activation (Chk1 for ATM and Chk2 for ATR) or via DNA damage response pathway. These cause DNA damage in telomeres where telomerase is reduced or the shelterin complex is impaired. [129] TRF2 can reduce the senescence set

point, defined as telomere length at senescence, from 7 to 4 kilobases that can cause a cell to initiate the senescence pathway. [130] TRF2 can also protect short telomeres and prevent checkpoint activation by more efficient formation of t-loop structures. [125] TRF2 also recruits another member of the shelterin complex – RAP1. Its in vivo role is not clearly understood but its overexpression can cause lengthening of telomeres. [131] It has been shown that RAP1 deficiency can be dispensable for telomere capping but leads to increased telomere recombination and fragility. [132] Also, RAP1 can bind to non-telomeric sites through the TTAGGG consensus motif, suggesting its role in transcriptional control. [132] Another shelterin component is POT1 (Protection of telomeres), and it can act as a telomerase dependent positive regulator of telomere length. POT1 binds to TPP1 at its carboxyl end and this union is essential for the localisation of POT1 in telomeres and also for the regulation of telomeric length interacting with telomerase. [133,134] TIN2 is an important mediator of TRF1 function [135] and has been shown to suppress telomere elongation in telomerase positive cells and could potentially control telomere length by modulating telomere function. [136] TPP1 is encoded by the ADC gene and there is evidence that it is required for telomerase recruitment to telomeres thereby preventing early occurrence of degenerative pathologies. [137] Thus it is clear that telomere function is not solely dependent on its length. The structure and level of expression of telomere binding proteins plays an important role in maintain telomere integrity and function.

Telomere position effect (TPE): refers to transcriptional silencing of genes adjacent to telomeres which can be the mechanism underlying telomere mediated senescence. [138] TPE has been best characterised in yeast Saccharomyces cerevisiae, where reversible silencing of a gene appeared to

be related to both to telomere length and distance from the telomere. [139] Also, marker genes inserted less than 4kb from telomeric repeats were frequently repressed and expressed late in S phase. [140] And hence it was postulated that telomere attrition in humans could effect the expression of various genes.

Transcriptional silencing near human telomeres was shown when luciferase reporters placed adjacent to telomeric repeats had a much lower expression than those placed at non-telomeric sites. Moreover, this reduced expression could be restored by using a histone deactetylase inhibitor, trichostatin A. Further support to these findings was provided by demonstrating that over-expression of hTERT in the telomeric clones resulted in telomeric extension and a 2 to 10-fold decrease in luciferase compared to control clones. Not only was the silencing effect of longer telomeres proven, but the magnitude of silencing correlated with the telomere length. [140]

Telomerase-Basic structure: is a ribonucleoprotein composed of a highly conserved reverse transcriptase (TERT) and a RNA primer (TERC) that provides the template for the synthesis of telomeric repeats at chromosomal ends. [141] The telomerase holoenzyme is believed to assemble in the cajal body where TERT and TERC form a ribonucleoprotein enzyme complex. [142] Although TERT and TERC can generate enzymatic activity in vitro, additional proteins are required for assembly and activity of telomerase in vivo. [142] These integral protein components of the heterogenous telomerase complex include dyskerin, NHP2, NOP10, pontin/reptin, Gar1 and TCAB1. [143] Dyskerin, pontin and reptin form a scaffold that recruits and stabilizes human telomerase RNA and assembles the telomerase ribonucleoprotein component. Once this complex is formed, pontin and reptin are believed to dissociate from the complex and release the catalytically active enzyme. [144]

Telomerase activity and telomere elongation by telomerase: telomerase activity varies markedly between different cell types. To determine this differential expression of telomerase, human foetal tissues of 8-21 weeks gestational age were assayed for telomerase activity. [145] All tissues expressed telomerase at the earliest ages examined. Lung, liver, spleen and testis maintained telomerase activity up till 21 weeks. Brain and kidney telomerase were detected till the 16th week and were undetectable thereafter. Cardiac tissue did not display any telomerase activity after 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 during gestational development is due to lack of active telomerase rather than the presence of an inhibitor. [145] From being highly expressed during embryonic development, telomerase activity is suppressed in somatic cells. Telomerase activity has been detected in highly proliferative cells like stem cells, progenitor cells, endometrium, lymphocytes, skin keratinocytes and in vast majority of cancers. [146]

Telomerase is capable of elongating telomeres and hence delaying the onset of senescence. In telomerase dependent replication, the RNA template of the telomerase complex binds to the 3' end of the telomeric DNA. This is followed by telomerase mediated G-strand elongation with the RNA moiety of the enzyme acting as the template and 3' end of chromosome acting as the primer. The complimentary C-rich strand is generated by lagging strand synthesis creating a single strand overhang. Telomere elongation by telomerase is tightly regulated during the cell cycle with telomeric repeats being added in the S phase and M phase. [147] Also, telomerase does not act on every

telomere in each cycle but characteristically shows an increased preference for telomeres as their lengths decline. [148]

1.4.3 Mechanisms of Telomere Shortening and Repair

Telomere shortening is a potential mechanism for a biological clock that determines cellular behaviour and is thought to trigger cell senescence and premature biological ageing. The two postulated mechanisms of telomere shortening are cellular replication and a more stochastic shortening secondary to external stimuli.

Telomere length and replicative senescence: the inability of DNA polymerases to replicate a linear DNA molecule to its very end, commonly known as the end replication problem, [108] leads to telomere shortening with every cell division. However, the rate per cell division probably is constant, but the rate as a function of population doubling may vary. It changes from cell to cell, from one division cycle to the next and as a function of external stressors. [149] Inflammation heightens the cellular turnover, which further exacerbates the age related telomeric shortening. This is a reflection of the replicative history of the cell and on this basis it has been suggested that telomere loss might serve as a mitotic clock.

The resulting telomere hypothesis of senescence states that (1) shortening of telomeres to some threshold value triggers senescence (2) telomeres shorten at a constant rate and so that telomere length is a faithful indicator of replicative history. [149]

Telomere length and stochastic shortening: The causal link between the gradual decrease in mean telomere length and senescence induction has been questioned by a number of observations. In their experiment on human fibroblasts infected with viral strains, Smith and Whitney (1980) found

increasing proportion of cells entering senescence at late passages and hence demonstrating bimodal nature of senescence. [150] However, the mean telomere length shortens in a linear fashion. [151] Also, in tissue cultures, senescence has been found to be induced with a mean telomere length much longer than that would be expected to trigger senescence mechanisms.

In addition to replicative shortening, sudden extensive shortening of single telomeres can take place by a more stochastic process that can be induced by various stimuli. Different mechanisms for this sudden shortening have been suggested. These include oxidative damage inflicting single stranded breaks in telomeres, replication slippage and unequal sister chromatid exchange and recombination causing circularisation and thus deletion of distal repeats. However, the predominant mechanism amongst these is exposure to oxidative stress. [152]

Oxidative stress can have a much greater and much faster impact on telomere shortening than the end replication problem alone. Telomeres are more susceptible to damage caused by oxidative stress due to the direct erosion of GGG repeats and also a negative impact on telomere length is observed due to inhibition of telomerase activity by oxidative stress. [153]

A combination of increased cell turnover secondary to systemic and local inflammation and the effect of oxidative stress and free radicals are thought to be the basis of cellular senescence and premature biological ageing that marks the process of atherosclerosis. This is represented by the telomere length, which registers the turnover rate of cells, a rate that is augmented by chronic inflammation and an increase in the cumulative oxidative burden.

1.4.4 Telomeres and Cardiovascular Disease

There is evidence to suggest that some types of heart disease may represent a disease of premature biological ageing and that telomere length, independent of chronological age, is predictive of increased risk of cardiovascular disease.

Telomere biology and coronary artery disease: telomere length in circulating leukocytes has been shown to have a strong association with CAD in diverse populations. [95-99] Whether telomere shortening is causal for CAD or just an epiphenomenon is debatable. However, studies have shown the existence of telomere shortening before manifestation of clinical disease and hence supporting the hypothesis that telomere shortening can be the primary cause leading to clinical expression of a disease process.

In cross-sectional studies, shorter mean telomere length in circulating leukocytes has been associated with triple vessel CAD [100] and premature MI. [96] Age and sex adjusted mean telomere length in cases has been shown to be significantly shorter (300bp) in cases with atherosclerosis than in healthy controls. [96] A large cross-sectional study compared the telomere lengths of 598 male patients with CAD with 653 age-matched controls across Europe. In addition, 413 coronary artery bypass graft patients and 2 groups of 461 patients with familial hypercholesterolemia (FH), of whom 162 had premature CAD, were recruited. It was reported that telomere length was significantly shorter in cases (7.85 kb) compared with control subjects (8.04 kb, $P < 0.04$). This was also true for the coronary artery bypass graft sub-study. In the FH patients, leukocyte telomere length was shorter in those with CAD compared with the non-CAD subjects. On the other hand, telomere length was not associated with any measured CAD risk factors except for age. These data confirm that subjects with CAD have shorter telomeres than control subjects,

and extends this to those with monogenic and polygenic forms of CHD, such as FH. [101]

Relationship between telomere biology and risk of CAD in patients with acute coronary syndrome has been shown in a large prospective study [102] In addition, telomerase activity in leukocytes has been associated with the presence of calcified atherosclerotic coronary plaque, and shown to be a predictor of plaque progression in the presence of short telomeres. [103] Also, shorter telomeres are associated with high-risk plaque morphology on virtual histology intravascular ultrasound, but not total 3-vessel plaque burden in CAD.[103] Monocytes with disrupted telomeres show increased pro-inflammatory activity, which is also seen in CAD patients, suggesting that telomere shortening promotes or is associated with high-risk plaque subtypes by increasing pro-inflammatory activity. [154]

These observations were not limited to circulating leukocytes and similar results have been replicated in coronary endothelial cells as well. Not only were the coronary artery endothelial cell telomeres significantly shorter in cases when compared to age matched controls ($p< 0.0001$) but within the CAD cases there was significant difference in the telomere lengths between the atherosclerotic and non-atherosclerotic regions of the coronary artery. [100] Similar increased rates of telomere attrition in atherosclerotic regions have also been shown in the aortic endothelium. [155]

The cause-effect relationship of CAD and telomere attrition is not very well established but there is increasing evidence that short telomeres precede the development of clinical disease and are not just the cause of CAD. In the West of Scotland Primary Prevention Study (WOSCOPS), the odds ratio for coronary events over the next 4.9 years was almost doubled in placebo treated

subjects in the lower two tertiles of baseline telomere length compared with the highest, while a study of 143 people over the age of 60 found that having shorter than average telomeres was associated with a more than threefold higher cardiac mortality over the next 8.9 years. Furthermore, mean telomere length has been shown to be shorter in asymptomatic young adults with a two-generational history of heart disease compared to healthy age-matched controls with no such family history. This supports the hypothesis that shorter telomere length is a primary abnormality predisposing to cardiovascular disease. [98]

Because telomeres shorten with age and the average amount of telomeres lost per year can be computed, another way of presenting the difference in telomere length in those with or without CAD (or those who will develop CAD) is the number of years of age to which this equates. [156] A difference of 8 to 12 years has been shown across several studies; implying that patients with CAD have telomeres equivalent in size to normal subjects who are 8 to 12 years older). [95,96,98] This observation is invaluable to the concept of premature biological ageing in patients with (or those who are likely to develop) CAD.

Telomere biology and other cardiovascular diseases: shorter telomeres have shown to be associated with cardiovascular diseases other than CAD. Shorter telomeres have been associated with pathophysiology of CHF and decreased renal function in subjects with heart failure. [157,158] In a large cohort of 620 patients with CHF derived from the MERIT-HF study and 183 age and gender-balanced controls, telomere length of white blood cells was also about 40% reduced in patients with CHF and related to the severity of disease. [159] Decreased left ventricular ejection fraction in the elderly, without evidence of

previous myocardial infarction, has also been associated with reduced telomere length. One standard deviation of shorter telomeres was associated with a 5% lower ejection fraction. [160]

Telomere biology and vascular tissue: Strong co-relation between the telomere lengths of circulating leukocytes and vascular endothelium has been shown in cohort studies. Shorter telomeres in coronary endothelial cells from patients with CAD than in subjects without CAD, [161] exaggerated telomere shortening in atherosclerotic plaques and at sites prone to atherosclerosis such as bifurcations has been shown. [155] There is also evidence that endomyocardial biopsies of subjects with dilated cardiomyopathy have more rapid cellular senescence and cell death associated with a telomere length reduction of 39% in comparison to normal hearts. [161]

Telomere biology and cardiovascular risk factors: Various cardiovascular risk factors have been associated with shorter telomere length in leukocytes, including obesity, smoking and type-two diabetes. [162,165] Gender is a known risk factor for coronary artery disease, with prevalence higher in men. In women psychological stress, obesity and smoking and low socioeconomic status [163-165] have been associated with shorter leukocyte telomeres. Many of these associations could reflect the effect of oxidative stress or chronic inflammation on telomere attrition. [166] Telomere length has also been associated with hypertension. For a given chronological age, men with shorter telomeres are more likely to have a higher pulse pressure. [163] However, adjustment for these risk factors does not have a significant impact on the association between shorter telomere length and CAD suggesting that the relationship is not just a reflection of the effect of these risk factors on telomere

attrition. Incidence of cardiovascular disease is influenced by ethnicity and telomere lengths differ among geographical locations. [97,167]

Telomere biology and ventricular arrhythmias: There have been no studies to date, exploring the association of telomere biology with ventricular arrhythmias. VAs post MI can be caused by a number of mechanisms with re-entry being an important one in chronic CAD. Re-entry mechanisms are related to significant electrical heterogeneity as a result of structural and/or electrical remodeling of the left ventricle. Post infarction areas, including regional and intramural fibrotic zones and scars, remain electrically unexcitable and may form local conditions for re-entry leading to sustained monomorphic VT. [168,169] The manifestations of CAD create distinct and time-varying changes in the myocardium that enhance the risk of VAs. Scar formation after MI is a major contributor to arrhythmia susceptibility. Peri-infarct cardiomyocytes have been shown to exhibit progressive shortening of action potential duration post MI, followed by a return to near-normal action potential over time. [170] Moreover, peri-infarct cells have been shown to have reduced side-to-side coupling. [171] Heterogeneities in action potential duration and altered coupling between infarct border zone tissue and surrounding myocardium create a region of conduction slowing susceptible to local conduction block necessary for re-entrant excitation. Alternatively, disordered initiation or propagation of cardiac action potentials can also be a result of functional changes in myocardial ion channels. Chronic oxidative stress often involves changes in the transcript and protein expression of cardiac ion channels/transporters that are required to maintain normal electrical functioning and intracellular ionic homeostasis – this is known as electrical remodeling. [172,173] This electrophysiological change produces a substrate that is vulnerable to malignant VAs.

With the current evidence, it is difficult to be certain of the role of telomere biology in arrhythmogenesis. However, if an association is established between systemic telomere biology and clinical cardiac arrhythmias, then this will require further study of the interaction of telomere biology and functional cardiac electrophysiology and cardiac remodeling post MI. Telomere shortening is felt to contribute to biological ageing through an increased vulnerability to oxidative stress and subsequent fibrosis secondary to reduced genomic stability. [174] Ventricular myocardial fibrosis is considered central for initiation and maintenance of re-entry VT through its role in promoting electrical heterogeneity. Telomeres may be seen to play a central role in this by virtue of poor myocardial repair process. Other mechanisms of telomere mediated VAs in this cohort could include telomerase mediated prolonged lifespan of inflammatory cells promoting ischaemia or prolonged lifespan of peri-infarct cardiac cells promoting anisotropy and arrhythmic potential. Comprehensive mechanistic studies will be required to investigate this further if an initial correlation between telomere biology and VA is established in this study.

1.5 Rationale, Aims and Hypothesis

This review has demonstrated that ICDs are being increasingly used in current practice and are the most effective intervention for the prevention of SCD in ischaemic cardiomyopathy patients. However, they are associated with morbidity and mortality. The key investigation guiding ICD prescription under current guidelines is LVEF. However, this risk stratification process has major limitations and needs improvement.

Telomere biology offers a promising approach to an improved risk stratification process for primary prevention ICD implants. Considering the

evidence discussed in the previous sections, demonstrating a relationship between telomere biology and cardiovascular disease and taking into account the mechanisms of telomere shortening and arrhythmogenesis, we hypothesised that:

Telomere shortening at the time of the index event (myocardial infarction) results in poor myocardial repair process and predisposes patients to greater arrhythmic tendency.

Hence, the load of short telomeres, mean telomere length and/or telomerase activity would be different in ischaemic cardiomyopathy patients with and without VA and would co-relate with the incidence of VA.

In order to test this hypothesis, we carried out a retrospective case-control study in patients with ischaemic cardiomyopathy and ICDs. This was to facilitate a comprehensive investigation of the characteristics of leukocyte telomere biology and their association with development of potentially fatal VA. The specific aims of the study were:

1. To acquire detailed characterisation of the **mean leukocyte telomere length, load of short telomeres and telomerase activity** in patients with ischaemic cardiomyopathy with primary prevention ICDs and correlate these findings with the incidence of previously documented VA requiring ICD therapies (as a surrogate marker of SCD).
2. To investigate genetic variation in **telomere related single nucleotide polymorphisms** and correlate these variations alone and in combination with telomere dynamics with the incidence of previously documented VA requiring ICD therapies (as a surrogate marker of SCD).

3. To investigate any association between **mean telomere length, load of short telomeres and telomerase activity** and other clinical variables including eGFR, BNP, NYHA class and LVEF.

Chapter 2 – METHODS

2.1 Introduction to the Methods

The materials and methods utilised in the research project are described in this chapter. All patients were recruited from the device clinic at St Bartholomew's hospital. I screened the clinical notes of 350 patients attending the device clinic to identify if they met the inclusion and exclusion criteria of the study. Of these, 140 patients were eligible to participate in the study and were invited to participate. I discussed the study with them via initial telephone contact and then at their clinic visit before obtaining informed consent. Following this, a detailed clinical phenotypic assessment was done including medical history, echocardiograms, ICD interrogation and blood tests as detailed in the next section. Blood samples were immediately transferred on ice to the laboratory.

The vast majority of experiments were performed in the Translational Medicine and Therapeutics Laboratory at the William Harvey Heart Centre, Charterhouse Square Campus, Queen Mary University of London. A small subsection of experiments were carried out at Department of Clinical Genetics, Vejle Hospital, Aarhus University, Denmark.

The experiments described in this thesis were performed on human DNA extracted from peripheral blood leukocytes. All DNA samples were stored in an anonymous manner at the Translational Medicine and therapeutics Laboratory at the William Harvey Heart Centre, Charterhouse Square Campus, Queen Mary University of London. Storage of samples and all procedures carried out on them were in accordance with the Human Tissue Act 2004.

I performed all experiments used in this research project. I received initial training in telomere experimental methodology from Dr Scott Brouilette, post-doctoral fellow, at the William Harvey Heart Centre in Professor Suzuki's

laboratory. I was trained for qPCR analyses for mean telomere length on DNA samples obtained from a separate clinical cohort (Genomic Advances in Sepsis). I performed qPCR measurements (in duplicate) on a total of 80 samples of which 30 samples were analysed by both Dr Brouilette and myself (to determine the inter-individual variability) to ensure accuracy of the technique learnt. The coefficient of variance for these samples was 11%. For the 90 samples analysed in this study (results presented in section 3.3.3) qPCR measurements were performed in duplicate by myself. The intra-assay coefficient of variation was 5.8%. Dr Laila Bendix, Vejle Hospital, Denmark; supervised the analysis of load of short telomeres (see section 2.7 and 3.3.5) using the Universal Single Telomere Length Analysis protocol.

2.2 Study Design and Patient Recruitment

This case-control observational study was carried out at St Bartholomew's hospital and William Harvey Research Centre. Ethical approval was obtained from the local research ethics committee. All patients gave written informed consent and were recruited from the ICD clinic at St Bartholomew's Hospital. Investigators were blinded to clinical history and all telomere analysis was carried out blinded to clinical data.

To be eligible for recruitment, patients had to meet all of the following criteria:

Inclusion criteria

- Caucasian ethnicity
- Previous MI with coronary artery disease demonstrated on angiography
- No evidence of reversible ischaemia on angiography or functional imaging
- Poor systolic function with Left Ventricular Ejection Fraction ≤ 35%

- ICD implanted for primary prevention (risk of sudden cardiac death due to ischaemic cardiomyopathy)

Exclusion criteria

- Age < 18yrs
- Carcinoma
- Chronic inflammatory disorders ascertained by patients medical history, white cell count and CRP levels
- Inability or unwillingness to sign consent form
- Life expectancy less than 6 months as a result of non-cardiac disease

2.3 Phenotypic Assessment and Investigations

I assessed all patients were in the device clinic at recruitment. All patients underwent:

Medical history: a detailed medical history was obtained to include identification of hypertension, diabetes mellitus, chronic renal failure, hypercholesterolemia, smoking status, family history of ischaemic heart disease, New York Heart Association Functional Classification (NYHA) and a full drug history.

Echocardiogram: a trans-thoracic echocardiogram to calculate left ventricular dimensions and LVEF by modified Simpson's method.

ICD interrogation: all patients recruited to the study had primary prevention ICD implants on the background of ischaemic cardiomyopathy. Details of the ICD implant were noted including type of device, pacing thresholds and programming criteria. Programmed ventricular arrhythmia zones were based on standard departmental criteria for primary prevention devices at the time of implant. The vast majority of patients had their devices programmed as a two zone device (VT and VF) with a mean lower rate of VT zone at 170 ± 2 bpm

(programmed ATP and shock therapies) and the VF zone (shock only) at 205 ± 2 bpm, reflecting practice at our institution at the time. Based on the above device programming criteria, potentially fatal VA was defined as sustained ventricular tachycardia at a rate > 170 bpm (and lasting greater than 30 seconds) or ventricular fibrillation requiring device therapy. Over the course of follow-up since device implant, a small proportion of patients had some device programming changes done based on their clinical need. Although these changes could potentially have an impact on the study results, this was accepted as a recognised limitation of this retrospective case-control study design and heterogeneity of the patients recruited.

ICDs were interrogated to identify recent arrhythmias and therapies delivered since the last clinic visit. ICD therapies served as a surrogate for potentially fatal VA (and hence SCD) and included both ATP and shock. All previous ICD clinic notes and device traces (since implant) were individually looked at to identify both appropriate and inappropriate therapies (ATP and shocks). Inappropriate and aborted shocks were recorded but did not constitute a surrogate marker for SCD and were not included in the study analyses.

Blood analyses: peripheral blood samples were taken for assessment of total serum cholesterol, lipid profile, white cell count, creatinine and estimated GFR (by the MDRD equation). Additional samples were taken for measurement of leukocyte telomere length & telomerase activity (in duplicate), load of short telomeres and plasma BNP.

2.4 Leukocyte Collection and Preparation

2.4.1 Reagents and Solutions

The following reagents were used, solutions prepared and utilized for this experiment:

1. Reagents
    a. RPMI 1640 media (Sigma R0883)
    b. Penicillin/Streptomycin, 100 x (Sigma P0781)
    c. L-Glutamine, 200mM (Sigma F2442)
    d. Dimethylsulphoxide, DMSO (Sigma D2650)
    e. Histopaque 1077 (Sigma H8889)
2. Solutions preparation
    a. Wash Mix: this solution was used to wash the cells (leukocytes) and also served as a base for preparation of the Freeze mix. It was prepared by adding 5ml of Penicillin/Streptomycin and 5ml of 200mM Glutamine to 500 ml of RPMI 1640 media.
    b. Freeze Mix: this solution was used for cryopreservation of the cells (leukocytes). It was prepared by adding 5ml of DMSO and 15 ml of Foetal Bovine Serum (FBS) to 30 ml of Wash Mix.

2.4.2 Isolation of Leukocytes

Whole blood was drawn into 4 x 6 ml of EDTA vacutainer tubes and processed within half an hour of venepuncture. 3ml of histopaque was added to each accuspin tube, which was then centrifuged in a bench centrifuge (Hettich EBA 20, UK) at 800 g RCF for 30 seconds. This resulted in the histopaque moving below the fret within the accuspin tube. The blood is mixed well by inverting the EDTA vacutainer tube a few times. 5ml of blood was then added to the accuspin tube in a laminar flow hood. The tube was then centrifuged at 2500 rpm for 20 min. This enabled the lymphocyte layer to be completely separated from the remaining blood.

2.4.3 Collection of Leukocytes

Using a 3ml Pasteur pipette, the uppermost layer of fluid (plasma) was carefully removed from the accuspin tube and discarded into a waste bottle containing disinfectant (Virkon, Sigma, UK). The lymphocyte layer was then transferred to a 15ml centrifuge falcon.

2.4.4 Cryopreservation of Leukocytes

14ml of pre-warmed Wash mix was then added to the 15ml falcon containing leukocytes. This was then centrifuged at 1000 rpm (210g RCF) for 10 min at room temperature. The supernatant was discarded into a waste bottle containing disinfectant (Virkon, Sigma, UK) after centrifugation. The cells were then re-suspended in 2ml of Freeze mix and aliquoted into cryovials. These were stored at -80°C for future use for DNA isolation.

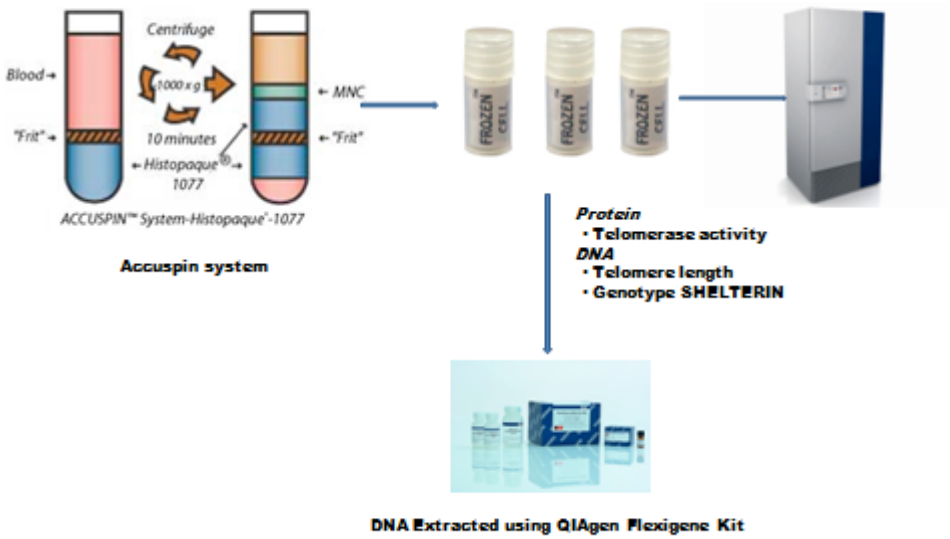

Figure 5: Buffy coat and DNA extraction

2.5 DNA extraction

2.5.1 Reagents and Solutions

The following reagents from the QIAgen Flexi Gene – QIAgen 51206 kit were utilized for this experiment:

1. Reagents
    a. Buffer FG1
    b. Buffer FG2/QIAgen Protease
    c. 100% Isopropanol
    d. 70% Ethanol
    e. Buffer FG3
2. Solutions prepared

    The BufferFG2/QIAgen Protease for different batch volumes of buffy coat (leukocyte volumes) are:

Total volume of Buffy Coat in batch (ml)	**1**	**3**	**5**	**10**	**30**	**50**	**60**
Volume of Buffer FG2 (ml)	1	3	5	10	30	50	60
Volume of QIAgen Protease (ul)	10	30	50	100	300	500	600

Table 2: volumes of Buffer FG2 (in ml) and QIAgen Protease (in µL) required to prepare different batch volumes (ml) of Buffy Coat reagent.

2.5.2 Cell Lysis

The frozen buffy coats were thawed in a 37°C bath with mild agitation for 30 min. 2 ml of buffy coat was added to a 15 ml falcon containing 5 ml of lysis buffer FG1. The solution was mixed by inverting and then centrifugation was carried out in a bench centrifuge (Hettich EBA 20, UK) for 5 min at 2000G RCF at room temperature. The supernatant was discarded in a waste bottle containing disinfectant (Virkon, Sigma, UK) and the falcon left inverted on an absorbent paper to get rid of any residual fluid, leaving a visible white pellet.

2.5.3 Protein Digestion

Denaturation buffer FG2 and QIagen protease (3ml/30µl) was added to the pellet and the falcon vortexed immediately (3-4 pulses for 5sec) till the pellet was completely homogenised. The falcon was inspected carefully to ensure complete homogenisation. If traces of pellet remained, then 300µl of denaturation buffer was added and the solution vortexed again. Further mixing was done by inverting the falcon thrice, following which it was incubated for 10 min at 65°C in a water bath. Protein digestion was indicated by change of solution colour from red to olive green.

2.5.4 DNA Precipitation

2ml of 100% isopropanol was then added and the solution mixed by inversion till precipitated DNA was visible as a white thread. Centrifugation was carried out in a bench centrifuge at 2000G RCF at room temperature for 3 min. The supernatant was poured off, taking care not to loose the pellet. The falcon was then inverted on absorbent paper to drain the residual fluid, taking care not to loose the pellet. The pellet was then washed by inversion in 2ml of 70% ethanol and vortexed for 5 sec. Centrifugation was carried out in a bench centrifuge at 2000G RCF for 3min at room temperature. The supernatant was discarded and the falcon inverted on absorbent paper for 10-15 min to drain the residual fluid. The DNA pellet was air dried for 5 min till all residual fluid had evaporated.

2.5.5 DNA Hydration

Up to 600µl of DNA hydration buffer FG3 was added to the pellet and the falcon vortexed for 5s at low speed. This was then placed in a water bath at

65°C for 1 hour to dissolve the DNA. The sample was then cooled at room temperature and stored at 4°C until DNA determination was carried out.

2.5.6 DNA Quantification

DNA was quantified in duplicate by spectrophotometry. Calibration of spectrophotometer was done by using hydration buffer FG3.

Nucleic acids have a peak absorbance in the ultraviolet range, at approximately 260 nm. Sample purity was assessed by using the 260/280 ratio – pure DNA has an absorbance 260/280 ratio of 1.8 – 1.9. Lower ratios are caused by substantial protein contamination whereas higher ratios indicate RNA contamination. Samples that were found to be too dilute were precipitated with ethanol and re-suspended in a smaller volume. [175]

2.6. Quantitative real time PCR analysis of mean telomere length

2.6.1 Quantitative Real time PCR Assay Principles

Real time PCR analysis

Polymerase chain reaction relies on the self-replicating nature of DNA, using short primers to initiate synthesis of a target sequence by a DNA polymerase. Parent strands are separated by heating the DNA, short primers are annealed and extended by DNA polymerase. The repetition of this process over numerous cycles (20-40), results in amplification of the original target sequence. In theory, doubling of the target sequence should occur after every replication. However, in practice, this only occurs till the reaction components are in excess, functional polymerase is present and the PCR products are being formed at a fixed rate (i.e. no primer-dimer or non-specific products are

accumulating). The point at which the amplification reaches a plateau (called ‘end point determination’) is used for quantification of PCR products.

Telomere PCR

When compared to conventional amplification, telomere PCR is challenging due to the repetitive nature of the telomeric repeats. The principle underlying telomere PCR was described by Cawthon in 2002 [176] in his novel method for estimating telomere length using real-time PCR and relative quantification. He determined the relative telomere lengths by measuring (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.

It is postulated that longer telomeres will have more potential primer annealing sites and hence there will be an increase in fluorescence and decrease in number of cycles needed to reach a given threshold compared to a shorter telomere.

Method of analysis

Analysis of the telomere PCR products can be done by a number of methods. Methods for relative quantification include 2-standard curves method, comparative CT method, Pfaffl and comparative quantification.

Comparative quantification (Corbett Research, Cambridge, UK) was employed for analysis of mean telomere length in this assay. It is one of the more recently developed methods that increases the accuracy of the assay and is widely used and published. Comparative quantification compares the relative expression of samples to a control sample in a run when a standard curve is not available.

The basic principle of comparative quantification is shown in Figure 6.

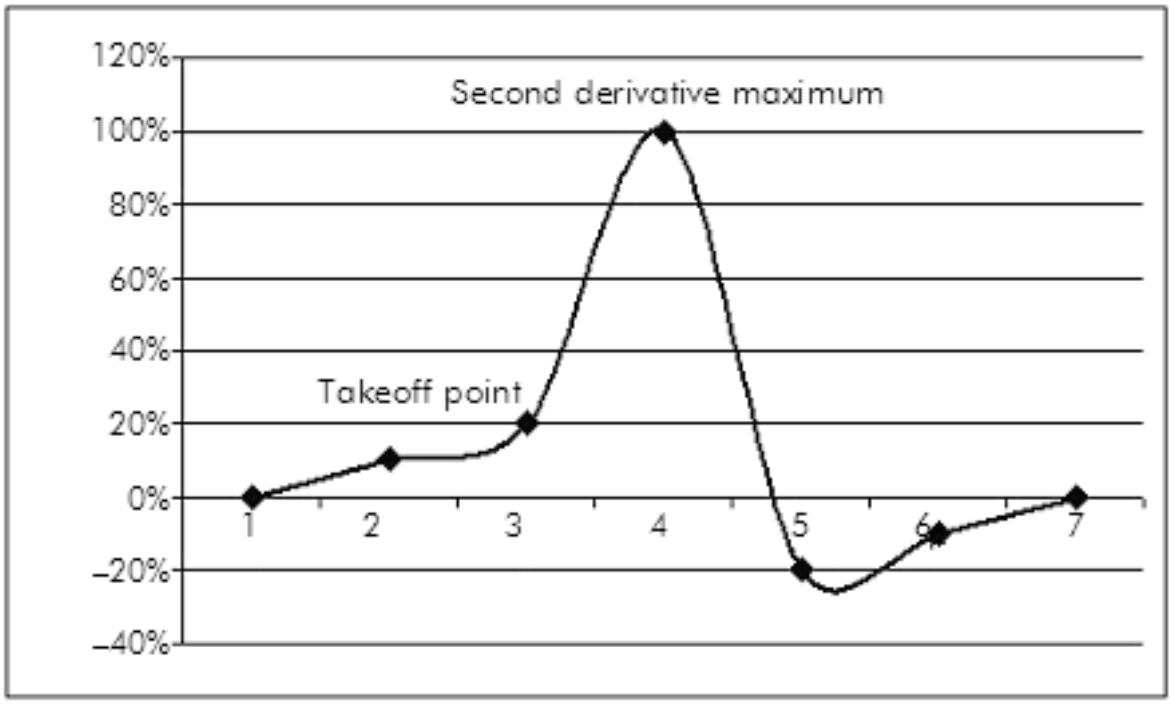

Figure 6: Graph showing comparative quantification analysis for telomere length measurement

[The take off point is determined for each sample by extrapolating back 80% from the peak reaction velocity]

(Adapted from Rotorgene version 6, Corbett Research)

It depicts the second derivative of an amplification plot, showing the relative positions of the second derivative peak and the take off point. The take off point is defined as the cycle at which the second derivative is at 20% of the maximum level and indicates the end of the noise and transition to the exponential phase. The second derivative of the amplification plot produces peaks corresponding to the maximum rate of fluorescence increase in the reaction. The amplification is an indication of the efficiency of the sample reaction. The amplification value of all samples is averaged to produce a mean amplification value. The relative concentration of each sample compared with the calibrator sample is calculated based on the take off point and reaction

efficiency. It calculates efficiency of every sample within a run rather than taking values from a set of standard curves. This increases the accuracy of the analysis compared to other methods.

An example of relative concentration analysis as per the software is as follows:

1. The take off point of each sample is calculated by looking at the second derivative peaks.
2. The average increase in raw data 4 cycles after the takeoff is calculated. This is the amplification value for the sample.
3. Outlier amplifications are removed to account for noise in background fluorescence.
4. The remaining amplifications are averaged. This is the average amplification.
5. The average takeoff point is calculated for each calibrator replicate.
6. The relative concentration for a sample is calculated according to this formula: Amplification^(Calibrator takeoff – Sample takeoff)

2.6.2 Reagents and Solution

The following reagents and solutions were utilized in this assay:

1. Reagents
    a. Primers: telomere (forward and reverse primers), 36b4 (forward and reverse primers), 100x conc (Sigma, UK)
    b. Quantifast SYBR Green PCR Mix: 2 x conc. Contains HotStar Taq Plus DNA Polymerase, SYBR Green PCR buffer, dNTP mix (dATP, dCTP, dGTP,dTTP) and ROX passive reference dye
    c. DEPC (diethylpyrocarbonate) treated water
    d. k562 cell line
2. Solutions

a. Primer working stock: dilute the master stock of primers with double distilled water to a working stock concentration of x 10.

2.6.3 Real Time Telomere PCR Assay

Leukocyte telomere length was measured with a quantitative real time PCR based technique that compares telomere repeat sequence copy number to single-copy gene (36b4) copy number in a given sample. This was done using the QuantiFast SYBR Green PCR mix (Qiagen, UK).

The DNA extraction and quantification was done as described before (see section 2.5). The QuantiFast SYBR green PCR master mix, primers and DNA were thawed on ice. Working stock of the primers was prepared as described above. Next, the reaction mix was prepared with 6uL of 2xQuantiFast SYBR Green PCR mastermix, 1.2uL each of forward and reverse telomere primers and 1.6uL of RNase-free DEPC water per reaction. The reaction mix was mixed thoroughly and aliquoted into PCR strip tubes. 2uL of template DNA at a concentration of 10ng/uL was added to each tube. All reactions were carried out in 12uL volumes. All DNA samples were run in duplicate. Non-template control (NTC) and calibrator sample k562 were also run in duplicate alongside the samples.

The samples were run in a 72 well rotor (Rotor Gene-Q, Qiagen). Cycling conditions were 95°C incubation for 10 min (activates DNA polymerase), followed by 20 cycles (for telomere) and 30 cycles (36b4) of 95°C for 15s (denaturation) and 58°C for 1 min (combined annealing/extension). This was followed by a melt ramp from 50°C to 99°C with the rise of temperature being 1°C with each step. 90s of pre-melt conditioning was carried out prior to first step, thereafter it was 5s for each step. The specificity of all amplifications was determined by melting curve analysis. 34 study samples, a calibrator

sample and one no-template control sample (all in duplicate) were processed per run.

The PCR reaction described above was carried out using both telomere and 36b4 specific primers in two separate runs. The telomere specific primers used were forward primer - 5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3', reverse primer - 5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3; the 36b4 primers used were forward primer - 5'CAGCAAGTGGGAAGGTGTAATCC3', reverse primer - 5'CCCATTCTATCATCAACGGGTACAA3'.

Analysis of PCR output was performed using Comparative Quantitation (Corbett Life Science Rotorgene analysis software version 1.7, Corbett Life Science, UK). The takeoff value of each sample was calculated based on the second derivative peak of the amplification plot, which represents the start of the exponential phase. The amplification efficiency was calculated for all the samples across the run and the mean amplification efficiency determined. The relative concentration of the sample was then calculated relative to the calibrator sample using the takeoff value and amplification efficiency. The same calibrator sample was used in all the runs to allow comparison of results across the runs. This process was performed for both the telomere (T) and 36b4 (S) assays and the telomere length expressed as the ratio of these (T/S), using mean data from duplicate runs.

2.7. Quantification of critically short telomeres by Universal STELA

2.7.1 Universal STELA Assay Principles

The Universal Single Telomere Length Analysis (U-STELA) assay enables the measurement of the load and distribution of the shortest telomere within an entire cell population. This is a modification of the original STELA assay, which measures the shortest telomeres on single chromosomes within a cell. As opposed to the original STELA, this assay can measure the load of short telomeres in a biological sample regardless of the specific chromosomal location of the shortest telomere. U-STELA assay principles are outlined in the figure below. [152]

The extracted DNA is digested with the help of restriction enzymes, which leave the same sticky overhang. The choice of these restriction enzymes is crucial as they determine the size of subtelomeric fragment, which is eventually included in the estimated telomere length. The combination of restriction enzymes used was MseI/NdeI as this mixture digests the DNA very proximal in the telomeric block, leaving at worst only a very short, although unknown, part of non-telomeric DNA in the final PCR product. However, there is a very small chance of the telomere being cut in the most centromeric part of the telomeric repeat block (1). The digestion step generates pieces of genomic DNA varying between 10-3000 bp, the vast majority of which have the same sticky overhang 5'-AT-3' at both ends (2). However, for every chromosome end, a fragment of DNA with the telomeric region including the 3'-overhang at one end and a smaller part of the sub-telomeric region with a 5'-AT-3' overhang at the other end, is also formed (3).

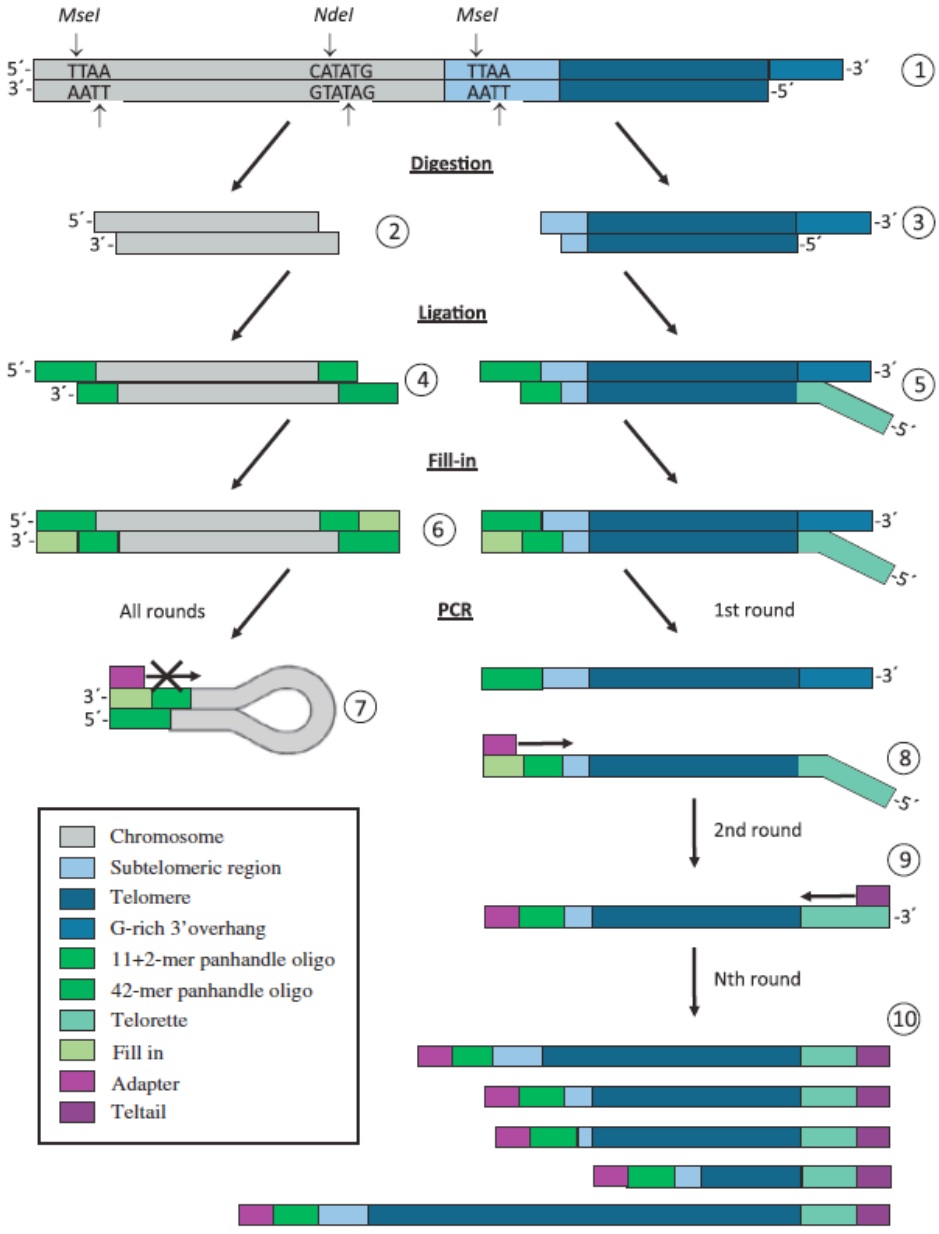

Figure 7: Image showing U-STELA assay principles

[Extracted DNA is digested by restriction enzymes MseI ⁄ NdeI (1). The digestion mainly generates pieces of genomic DNA of 10–3000 bp, the great majority of which has the same sticky overhang 5'-AT-3' at both ends (2). However, for every chromosome end a fragment of DNA with the telomeric region including the 3'-overhang at one end and a smaller part of the subtelomeric region with a 5'-AT-3' overhang at the other end, is also formed (3). The next step is a ligation-based step, in which two specially designed oligos (42-mer and 11 + 2-mer panhandle oligo) are annealed and ligated to the proximal overhang and to both ends of non-telomeric fragments (for oligo sequences see Table 1). These two oligos are designed so that they

spontaneously anneal, forming a two-base sticky overhang complementary to the overhang formed by the digestion. The other end of the paired oligos is a long, single-stranded and GC-rich overhang (4 and 5). The third step is another ligation step. An oligo (telorette) is annealed to the G-rich 3'-overhang of the telomeric repeat (5). This oligo consists of seven bases complementary to the telomere repeat sequence and a tail of 20 non-complementary nucleotides. After annealing the telorette is ligated to the 5'- end of the C-rich strand of the telomere. Finally a fill-in step is required (6) so that the GC-rich proximal overhang becomes double stranded, hereby serving as template for the upstream PCR primer (the adapter primer). In the first step of the PCR all DNA pieces are denatured. When the temperature is lowered for the annealing step, two things can happen. For the intra-genomic fragments where the upstream sequence is ligated to both ends, the complementary ends will anneal to each other, forming a panhandle, which will be relatively stable due to a high melting temperature of the panhandle sequence. A PCR, based on intragenomic fragments as template, will therefore be suppressed (7). For the telomeric fragments (8), the adapter will anneal to the filled-in part of the upstream sequence, thereby initiating PCR. In the following PCR cycles the teltail primer will be able to anneal to the PCR product obtained by the adapter primer (9), thereby producing telomeric products of different lengths reflecting the lengths of the individual telomeres (10).]

(Adapted from Bendix et al, 2010) ***[152]***

The next step is a ligation-based step, in which two specially designed oligos (42-mer and 11 + 2-mer panhandle oligo) are annealed and ligated to the proximal overhang and to both ends of non-telomeric fragments. These two oligos are designed so that they spontaneously anneal, forming a two-base sticky overhang complementary to the overhang formed by the digestion. The other end of the paired oligos is a long, single-stranded and GC-rich overhang (4 and 5).

Following this, another ligation step is carried out. An oligo (telorette) is annealed to the G-rich 3'-overhang of the telomeric repeat (5). This oligo consists of seven bases complementary to the telomere repeat sequence and a tail of 20 non-complementary nucleotides. After annealing the telorette is ligated to the 5'- end of the C-rich strand of the telomere.

Finally a fill-in step is required (6) so that the GC-rich proximal overhang becomes double stranded, hereby serving as template for the upstream PCR

primer (the adapter primer). In the first step of the PCR all DNA pieces are denatured. When the temperature is lowered for the annealing step, two things can happen. For the intra-genomic fragments where the upstream sequence is ligated to both ends, the complementary ends will anneal to each other, forming a panhandle, which will be relatively stable due to a high melting temperature of the panhandle sequence. A PCR, based on intragenomic fragments as template, will therefore be suppressed (7).

For the telomeric fragments (8), the adapter will anneal to the filled-in part of the upstream sequence, thereby initiating PCR. In the following PCR cycles the teltail primer will be able to anneal to the PCR product obtained by the adapter primer (9), thereby producing telomeric products of different lengths reflecting the lengths of the individual telomeres (10).

2.7.2 Reagents and Solutions

The following reagents and solutions were utilised in this assay:

1. Reagents
    a. Restriction enzymes M*se*I and N*de*I; H*inf*I and R*sa*I, 40U/uL (Roche Applied Sciences)
    b. TE Buffer, 20x conc (10mM TRis-Hcl, 1mM EDTA, pH 7.5) (Invitrogen)
    c. Quanti-iT Picogreen dsDNA reagent (Invitrogen)
    d. Dimethylsulphoxide, DMSO
    e. Lambda DNA standard, concentration 100ug/ml (Quanti-iT Picogreen dsDNA kit)
    f. Oligonucleotides (50uM)
        - 11+2 mer panhandle (5'TACCCGCGTCCGC3')

- 42 mer panhandle (5'TGT AGC GTG AAG ACG ACA GAA AGG GCG TGG TGC GGA CGC GGG3')
- Telorette 3 (5'TGC TCC GTG CAT CTG GCA TCC CTA ACC3')

g. NE Buffer 2 (New England Biolabs)

h. ATP (New England Biolabs)

i. T4 DNA Ligase, 20U (New England Biolabs)

j. Diethylpyrocarbonate treated water (DEPC)

k. Primers

- Adapter primer, 0.1uM (5'TGT AGC GTG AAG ACG ACA GAA3')
- Teltail primer, 0.1uM (5'TGC TCC GTG CAT CTG GCA TC3'DIG)

l. Bovine Serum Albumin (BSA, New England Biolabs)

m. Failsafe Buffer H (Epicentre, VWR, Denmark)

n. Failsafe Enzyme (Epicentre, VWR, Denmark)

o. Failsafe PCR Premix H (Epicentre, VWR, Denmark)

p. Digestion Buffer 10x conc (Roche Applied Science)

q. Control DNA, low and high, 50uL (Roche Applied Science)

r. DIG Molecular weight marker (Roche Applied Science)

s. Loading buffer, 5x conc (Roche Applied Science)

t. DIG Easy Hybridisation granules (Roche Applied Science)

u. Telomere hybridization probe (Roche Applied Science)

v. Washing buffer, Maleic acid buffer, Blocking buffer 10xconc (Roche Applied Science)

w. Anti-DIG-AP antibody, 0.75 U/uL (Roche Applied Science)

x. Detection Buffer, 10x conc (Roche Applied Science)

y. Substrate, Denaturation and SSC solutions (Roche Applied Science)

2. Solutions Prepared

a. TE Buffer working solution: prepare1 x TE buffer working solution by diluting the concentrated buffer 20 fold with sterile, distilled, DNAse free water
b. Picogreen dsDNA reagent: add 200 fold dilution of concentrated DMSO solution in TE Buffer. Add 100uL of Quanti-iT Picogreen dsDNA reagent to 19.9ml TE.
c. Lambda DNA standard, working solution: dilute original lambda DNA standard (100ug/ml) by 50 fold in TE buffer to achieve a concentration of 2ug/ml
d. Dig Easy Hyb working solution: re-constitute Dig Easy Hyb granules with 64 ml autoclaved, redistilled water and incubate at 37°C until complete re-constitution. Solution should be prepared several hours before use
e. SSC working solution: dilute the 20 x SSC, 1:10 with double distilled autoclaved water to attain a concentration of 2 x SSC
f. Blocking solution: dilute the 10 x blocking buffer, 1:10 with maleic acid buffer
g. Anti-DIG-AP working solution: spin the Anti-DIG-AP vial for 5 min at 13000 rpm before use. Dilute with blocking solution to a final concentration of 75mU/ml (1:10,000)
h. Detection Buffer: dilute the 10 x detection buffer, 1:10 with autoclaved, double distilled water

2.7.3 DNA Digestion with Restriction Enzymes

DNA was extracted from buffy coats as described before. 50 patient samples were used per run with a DNA concentration of 10ng/uL. 2 positive controls and 2 negative controls were also run in the same plate. DNA digestion was carried

out with 1:1 mixture of restriction enzymes M*se*I and N*de*I (Roche Applies Sciences) and incubated at 37°C for 2 hours which was followed by inactivation at 65°C for 20 min.

The quality of DNA digestion and integrity of the initial DNA template were tested using agarose gel electrophoresis. 10uL of the digested DNA template was run alongside 10uL of the corresponding non-digested template and then visualised with a nucleic acid stain.

The digestion was confirmed to be adequate if the digested DNA appeared as a smear below 600bp. The original intact DNA template should be seen as a single compact band migrating at 23kb.

2.7.4 DNA Template Quantification

The samples were quantified again with Quant-iT Picogreen dsDNA assay (Invitrogen). The picogreen reagent mix was prepared with TE buffer.

The DNA standard curve was generated using lambda DNA standard provided in the kit. A range of standards was obtained with different concentrations. These were obtained by diluting the original lambda standard with double distilled water. The samples were analysed in triplicate, the DNA lambda standard in duplicate and BLANK sample (TE buffer) in duplicate.

100uL of working solution picogreen mix to each well. The reagents were mixed well and incubated at room temperature for 2-5 min away from light. Following this, the sample fluorescence was measured using a fluorescence microplate reader at standard fluorescein wavelengths (excitation 480nm, emission 520 nm). The fluorescence value of BLANK was then subtracted from that of each of the samples. The DNA concentration of the samples was determined based on the standards used and the samples were normalized to 2ng/uL.

2.7.5 Ligation with Linker Oligomers and Primers

8ng of digested DNA was used as template. The oligonucleotides mix containing 50umol of 42-mer and 50umol of 11+2-mer was added to the DNA template in a total volume of 10uL. The mixture was then ramped down from 65°C to 16°C over 1 hour. This was followed by a ligation step with 20U T4 DNA ligase (New England Biolabs) in a mixture containing 1 x NE Buffer2 and 1x ATP. This mixture was left to ligate overnight at 16°C.

The samples were heated to 35°C and a second ligation step was carried out with 20U of T4 DNA ligase (New England Biolabs), 10^{-3} uM telorette-3, 1 x NE Buffer2 and 1x ATP. After another 12 hours of ligation and 20 min of inactivation at 65°C, the ligated products were diluted with sterile autoclaved water to a final concentration of 40pg/uL.

2.7.6 Polymerase Chain Reaction

Polymerase chain reaction was then performed using the two ligated oligonucleotides as targets for PCR. The concentration mix comprised of 80pg of ligated DNA, 1X Failsafe PCR PreMix H (Epicentre, VWR, Denmark), 0.1µM primers (teltail and adapter) and 1.25 U Failsafe enzyme (Epicentre, VWR, Denmark), made up to a volume of 12uL. The reactions were carried out in a Hybaid Thermocycler (Thermo Electron) with the first cycle at 68°C for 5 min constituting the fill-in step. The second cycle was at 95°C for 2 min. This was followed by 26 cycles at 95°C for 15 sec, 58°C for 30 sec and 72°C for 12 min. The last cycle was at 72°C for 15 min, following which the samples were held at 4°C.

2.7.7 Southern Blotting and Detection

STELA products were detected by Southern Blotting using TeloTAGGG telomere length assay (Roche Applied Sciences, Denmark) according to the manufacturer's manual.

Separation of the DNA products was done by agarose gel electrophoresis. A 0.8% agarose gel was prepared in 1 x TAE buffer using highly pure, nucleic acid grade agarose – Agarose MP. 4uL of DIG molecular weight marker was mixed with 12uL of nuclease free water and 4uL of 5x loading buffer. The samples were then loaded on to the gel along with the DIG molecular weight marker. The gel was then run at 5V/cm in 1 x TAE buffer.

For blotting, the gel was immersed in HCl and then rinsed with water twice. The gel was then submerged in denaturation solution. This was rinsed with water again and then submerged in neutralization solution. Following this, the STELA products were transferred onto a positively charged nylon membrane by southern blot vacuum transfer using VacuGene^{TM} *XL* Vacuum Blotting System (GE Healthcare). The transferred DNA was fixed with UV crosslinking and the membrane washed with 2 x SSC solution.

The blotted STELA products were then hybridized overnight to a DIG-labeled probe specific for the telomeric sequence. These were subsequently incubated with a DIG-specific antibody coupled to alkaline phosphate (Roche Applied Sciences).

Finally, after the addition of detection buffer and substrate solution, the telomere probe was visualized using the chemiluminescent substrate *CDP-Star* (Roche Applied Sciences), and the chemiluminescence signal was detected using *BioSpectrum Imaging System* (UVP, AH Diagnostics).

2.7.8 Calculation of Load of Short Telomeres

The size of the PCR products was calculated relative to a DIG-labeled molecular weight marker (Roche Applied Sciences) using *VisionWorks LS Acquisition and Analysis Software* (Ultra-Violet Products Ltd, AH Diagnostics).

The number of PCR products with a length less than 1500 base pairs (bp) were counted and calibrated based on the PCR template concentration. The load of short telomeres was presented as the number of telomeres below a length of 1500 bp per genome equivalent of template DNA.

2.8. Quantification of Telomerase Activity by Telomere Repeat Amplification Protocol

2.8.1 TRAP Assay Principles

Quantification of telomerase activity was done by the Telomere Repeat Amplification (TRAP) protocol, which allows highly specific amplification of telomerase mediated elongation products combined with non-radioactive photometric detection following an Enzyme Linked Immunosorbent Assay (ELISA) protocol.

The TRAP assay consists of two steps (1) Elongation/amplification (2) Detection of products by ELISA.

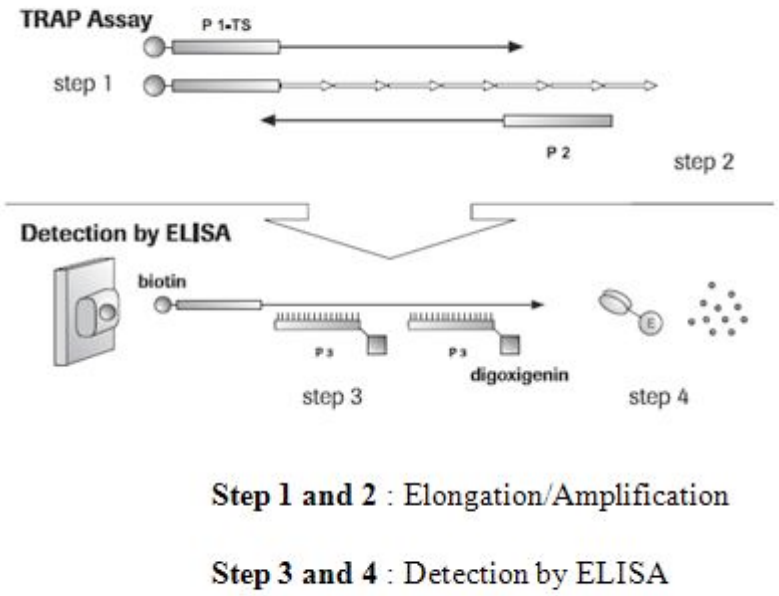

Figure 8: Image showing principle underlying Telomerase ELISA assay

[In step 1 (elongation), telomerase adds telomeric repeats to the 3' end of the biotin labelled synthetic P1-TS primer.In the second step (amplification),the elongated products are amplified by PCR using the P1-TS and P2 primersgenertaing PCR products with the telomerase specific 6 nucleotide increments. Step 3 and 4 include detection if these products by ELISA]
(Taken from TeloTAGGG telomerase PCR ELISA manual) [177]

In the first step, telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin labeled synthetic P1-TS primer based on the telomerase content of the sample (number of telomeric repeats α telomerase activity). These elongation products are then amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase specific 6 nucleotide increments.

In the second step, an aliquot of the PCR product is denatured and hybridised to a digoxigenin (DIG) labeled, telomeric repeat-specific detection probe. The resulting product is immobilized via the biotin labeled primer to a streptavidin coated microplate. The immobilized PCR product is then detected

with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. The probe is then visualised by virtue of peroxidise metabolising TMB to form a coloured reaction product. [177]

2.8.2 Reagents and Solutions

The following reagents from the Quanti-Fast Picogreen (QiaGen, UK) and Telo TAGGG Telomerase PCR ELISA (Roche,UK), were used in this assay:

1. Reagents
    a. Trypsin
    b. Phosphate buffered saline (PBS)
    c. Lysis reagent

        Is used for preparation of cell extracts from cell cultures.
    d. Reaction mixture

        Is a tris-buffer which contains telomerase substrate, primers, nucleotides and Taq polymerase. It also contains biotin labeled P1-TS primer and P2 primer. This is used for the telomerase mediated primer elongation and PCR amplification.
    e. Denaturation reagent

        Is a sodium hydroxide (0.5%) solution
    f. Hybridization buffer

        Contains a DIG-labeled detection probe complementary to the telomeric repeat sequence.
    g. Washing buffer
    h. Anti-DIG-POD (Anti-digoxigenin-peroxidase)

        It is a lyophilized stabilized polyclonal antibody from sheep.
    i. Conjugate dilution buffer
    j. TMB substrate solution

Contains the POD substrate 3,3',5,5'-tetramethyl benzidine

k. STOP reagent

Contains sulfuric acid (<5%)

l. Positive control extract

Is a cell extract prepared from lyophilized immortalized telomerase-expressing human kidney cells (293)

m. Microplate (MTP)

Is a strip frame with 12x8 well modules pre-coated with streptavidin and post-coated with blocking reagent with a desiccant capsule.

2. Solutions Preparation

a. Washing buffer x1

An appropriate volume of washing buffer (10x conc) was diluted with autoclaved double distilled water (1:10) and mixed thoroughly. The diluted solution is stable for 1 month at temperatures of 2-8°C.

b. Anti-DIG-POD stock solution

The Anti-DIG-POD lyophilisate was re-constituted in 240uL of autoclaved double distilled water. The resultant solution had an antibody concentration of 0.5U/ml. This solution is stable for 6 months at a temperature of 2-8°C.

c. Anti-DIG-POD Working Solution

This was prepared by diluting an appropriate amount of the re-constituted anti-DIG-POD stock solution with conjugate dilution buffer to a final concentration of 10mU/ml. This solution cannot be stored and should be prepared immediately before use.

d. Positive Control Cell Extract

The lyophilized cell extract was re-constituted on ice with 20uL autoclaved double distilled water and mix thoroughly. The re-constituted solution had a concentration of $1x10^3$ cell equivalents per microliter. The solution was then dispensed in into 1-3uL aliquots. This solution cannot be stored and needs to be prepared immediately before use.

2.8.3 Cell Counting with Automated Cell Counter

The frozen buffy coats were thawed at 37°C for 5 min. These were vortexed at a slow speed and kept on ice. 20uL of cells from buffy coat were suspended in 20uL of trypsin in an ependorff. This was vortexed at a slow speed for a couple of seconds. These cells were then analysed using a Countess Automated Cell Counter (Invitrogen, UK). 10uL of the above suspension was added to a glass slide specific to the cell counter machine (Countess Cell Counting Chamber Slides / Invitrogen, UK, C10228). On some occasions, the suspension was found to be too concentrated for cell count. This was then diluted with PBS 1:5 or 1:10, following which the cells were trypsinised for a repeat cell count. An automated cell count was obtained and the number of live cells and viability recorded.

2.8.4 Generation of Negative Controls

Heat inactivated negative controls were generated for this assay. Cell solution equivalent to 2 x 10^5 cells per single reaction were transferred to a new ependorff. Centrifugation was carried out at 3000G RCF at a temperature of 4°C for 10 min. The supernatant was aspirated in the laminar flow hood with a vacuum tip. The resulting pellet was then re-suspended in 500uL of PBS (pre-chilled on ice).

Again, centrifugation was carried out at 3000G RCF at a temperature of 4°c FOR 10 min. The supernatant was discarded and the pelleted cells were re-suspended in 200uL of lysis solution (pre-chilled on ice). Homogenous suspension was ensured by pipetting the solution a few times, followed by slow vortex for a few seconds. These were then left to incubate on ice for 30 min. Following this centrifugation was carried out at 16000G RCF at a temperature of 4°C for 20 min. 175 uL of the resultant supernatant was transferred to a fresh ependorff.

10uL of each sample was transferred to a 96 well plate. These samples were then incubated at 85°C for 10 min. Negative controls were thus generated by heat inactivation of the proteins.

2.8.5 Telomere Repeat Amplification Protocol (TRAP reaction)

The 96 well plate with the negative controls was then cooled on ice. 10uL of samples were then transferred on to the same plate as depicted in the plate layout below.

NC	**NC**	**NC**	**NC**		**S**	**S**	**S**	**S**			

Table 3: 96-well plate layout for negative controls (NC) and samples (S)

The reaction mix is thawed on ice and the positive controls re-constituted with 20uL of diethylpyrocardbonate (DEPC) treated water (Invitrogen, UK). The amount of reaction mix and DEPC treated water required for one reaction plate was calculated based on number of samples, keeping the DNA amount fixed at

3uL. These proportions were calculated for 28 samples, 28 negative controls and 2 positive controls for one reaction plate as shown in table 4.

	x 1 (μg)	**x 60**
Reaction Mix	25	1500
DNA	**3**	
dH_2O	22	1320
Total	**50**	

Table 4: Table showing quantities of reaction mix, DNA and distilled water (all in μL) required for setting up PCR reaction for 1 and 60 samples

Hot start PCR was then carried out using PCR system and a combined primer elongation and amplification reaction performed in a thermal cycler. For the first cycle the samples are kept at 25°C for 10 min to enable primer elongation. The second cycle was designed for telomerase inactivation wherein samples were kept at 94°C for 5 min. Amplification of the samples was then carried out over the next 30 cycles with programmed temperature variation cycling through 94°C for 30s, 50°C for 30s and 72°C for 90s to facilitate denaturation, annealing and polymerization respectively. The final cycle holds the samples at 72°C for 10 min. This is summarised in table 5.

	TIME	**TEMPERATURE**	**CYCLES**
Primer elongation	10 min	25°C	1
Telomerase inactivation	5 min	94°C	1
Amplification: Denaturation Annealing Polymerisation	 30s 30s 90s	 94°C 50°C 72°C	 1-30
	10 min	72°C	1
		4°C	

Table 5: Table showing various steps of TRAP reaction elongation/amplification protocol

[Primer elongation and PCR cycling steps with optimal conditions – temperature and number of cycles]

The 96 well plate was transferred on to ice after holding it at 4°C in the PCR machine.

2.8.6 Hybridisation and Detection by Enzyme Linked Immunosorbent Assay

Following PCR, 5µl of amplification product (samples in duplicate) and 20µl denaturation solution was added in each well of a fresh 96-well plate. These were then incubated at room temperature for 10 min. 225uL of hybridization buffer was added to each well. Following this, 100uL of each sample was transferred to a pre-coated MP module. The plate layout is shown below.

S	SD	NC	S	SD	NC	S	SD	NC	S	SD	NC
											PC
											PC

S: sample
SD: sample duplicate (technical replicate)
NC: negative control
PC: positive control

Table 6: MP Module layout

The next step involved shaking the MP module at 300RPM at 37°C for 2 hours. The hybridization solution was then removed completely by washing with wash buffer. This was done over three washes with 200uL of wash buffer per well for 30s per wash. 100µl of Anti-DIGPOD was added to each well. The MP module was covered with foil and then incubated at room temperature for 30 min while shaking at 300RPM. Further five washes were carried out with 200uL of wash buffer per well for 30s per wash. 100µl of substrate solution (pre-warmed to

room temperature) was added to each well. The MP module was then covered with foil and incubated at room temperature for 20 min while shaking at 300RPM. A colour change to blue, relative to positive controls, in the samples was observed. At this point, 100uL of STOP solution was added to each well and a colour change to yellow was observed in each well. The telomerase activity was measured on the ELISA plate reader at an absorbance of 450nm and a reference filter of 690nm within 30 min of addition of the STOP solution.

2.9. SNP Genotype analysis by Taqman assay

2.9.1 Taqman SNP Genotyping Assay Principles

Taqman genotyping probes are used for detection of single nucleotide polymorphisms by increasing the specificity of PCR reactions. Its basic principle relies on the 5'-3' nuclease activity of the enzyme Taq-polymerase and fluorophobe based detection.

The genotyping assay contains two primers for amplifying the polymorphic sequence of interest and two fluorescent probes with a minor grove binder (MGB) at the 3' end of each probe. The two primers target the region that flanks the SNP of interest. The MGB probe contains a reporter dye at the 5' end – VIC dye linked to allele 1 probe and FAM dye linked to allele 2 probe. A non-fluorescent quencher (NFQ) is also present at the 3' end of each probe. The NFQ prevents liberation of the reporter fluorescence if the probe is not degraded. The MGB stabilises the double stranded matrix formed between the probe and the target.

During the PCR reaction, each taqman probe anneals specifically to its complementary sequence between the forward and reverse primer sites as shown in the figure below. While the oligonucleotide probe is intact, the

proximity of the reporter dye to the quencher dye results in quenching of the reporter fluorescence primarily by Forster type energy transfer (FRET).

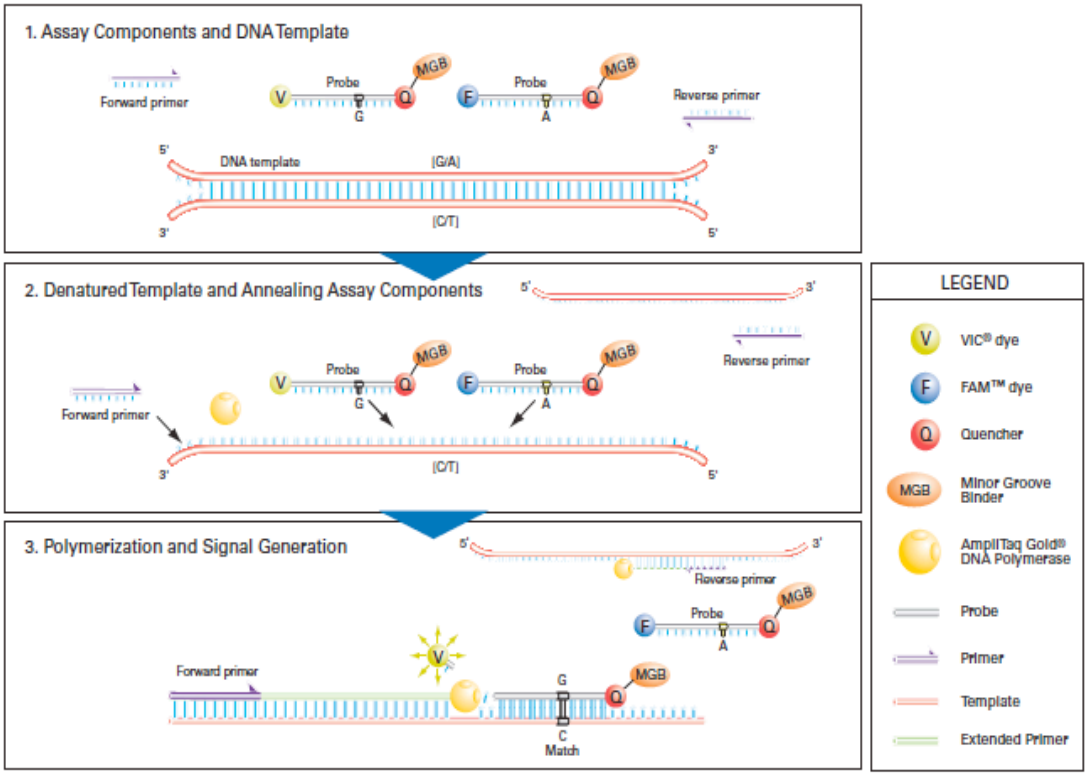

Figure 9: Image depicting the 5' Nuclease assay process

[Various steps of the 5'nuclease assay as described in the text are shown here] (Taken from QiAGEN SNP Probe manual) [178]

The primers bound to the template DNA are extended by the DNA polymerase. The DNA polymerase cleaves only those probes that are hybridized to the target. This cleavage separates the reporter dye from the quencher dye, which results in increased fluorescence by the reporter. This increase in fluorescence signal only occurs when probes that have hybridized to the complementary sequences have cleaved. And hence, the fluorescence signal generated by PCR amplification indicated which alleles are present in the sample.

A substantial increase in VIC-dye fluorescence only indicates homozygosity for allele 1. FAM-dye fluorescence increase indicates homozygosity for allele 2. Whereas, both VIC- and FAM-dye fluorescence indicates allele 1 and allele 2 heterozygosity. [178]

2.9.2 Reagents and Solutions

The following reagents and solutions (Taqman SNP genotyping assays, Applied Biosystems) were used in this assay:

1. Reagents
    a. Taqman genotyping assay: two MGB probes (one probe labeled with VIC dye detects allele 1 sequence, the other probe labeled with FAM dye detects the allele 2 sequence) and two primers for amplifying polymorphic sequence of interest.
    b. Taqman genotyping master mix
    c. TE buffer (10mM Tris-HCl , 1mM EDTA, pH 8, made using DNase-free, autoclaved, double distilled water)
    d. DEPC (diethylpyrocarbonate) treated water
2. Solutions
    a. SNP genotyping assay working solution: dilute the 80 x SNP genotyping assay with TE buffer to 20 x working stock. Multiple aliquots of the diluted SNP genotyping assay can be stored at -25°C

2.9.3 Quantifying Genomic DNA

The DNA was quantified as described before (see section)

2.9.4 Reaction Mix and Wet DNA Platform Preparation

The reaction mix was prepared using 0.5 uL of 20 x SNP Genotyping probe and 5uL of 2 x PCR Mastermix. The genotyping probe was vortexed slowly for

a minute before addition to the mastermix to ensure homogenous mixing. The DNA samples were diluted with DEPC water to deliver a final concentration of 20 ng. The mastermix was aliquoted into 0.1 ml strip tubes (72-well Rotor Gene-Q, QiAgen), following which genomic DNA was added to strip tubes. Two no template controls (NTC) were also included. All reactions were carried out in a final volume of 10uL. All pipetting was carried out in a laminar flow hood to avoid any cross contamination. Once the DNA platform is ready it was centrifuged briefly to spin down the contents and eliminate any air bubbles.

2.9.5 PCR Amplification

PCR was carried out in a real time PCR thermal cycler (Rotorgene, QiAgen, UK). The samples were initially incubated with temperature increased to 95°C and held at that temperature for 10 min. The polymerase is activated by this heating step. This was followed by 40 cycles of PCR at 92°C for 15s (denaturation) and 60°C for 90s (anneal/extend).

2.9.6 Allelic Discrimination and Scatter Plot Analysis

After PCR amplification, allelic discrimination read and analysis was performed using the Rotor Gene Q software (Qiagen). Allelic discrimination uses real-time kinetic from two or more channels to genotype samples. Within the SNP genotyping tool, the allelic discrimination software was used. Both Cycling A Green and Cycling A Yellow channels were selected and genotypes assigned to each channel. Automatic allele calls were made by the software, which were subsequently verified. Allelic discrimination analysis was performed based on set thresholds.

Scatter graph analysis was also performed. This enables genotyping based on relative expression of amplification plots across two channels. The display is normalized to account for different fold increases in each channel

and log transformed to accentuate the differences in expression between samples. Unlike allelic discrimination, genotype here was determined based on regions defined by a scatter graph rather than from a single threshold.

Chapter 3 – ASSOCIATION OF TELOMERE DYNAMICS WITH VENTRICULAR ARRHYTHMIAS

3.1 Introduction and Aims

As discussed in Chapter 1, ICDs have a clear survival benefit in high-risk patients with severely impaired left ventricular function post MI. [1,19,20] However, ICDs are associated with morbidity and mortality. Importantly, 67% of the patients never receive an appropriate shock after ICD implantation under the current guidelines, suggesting a need for better risk stratification. [80,81]

Telomere length and telomerase activity in leukocytes have recently been shown to correlate with biological ageing, health status and pathogenesis/prognosis of several types of cardiovascular diseases. Telomere biology has the potential of identifying accelerated biological ageing distinct from chronological ageing and help predict the occurrence of cardiovascular events. It has been associated with CAD in diverse populations, triple vessel CAD, premature myocardial infarction and acute coronary syndromes. [94-97] Telomere shortening has also shown to correlate strongly with the presence of cardiovascular risk factors (including obesity, smoking and type two diabetes), decreased renal function in subjects with heart failure and presence of atherosclerotic plaques in vascular tissues. [99,101,157,158,165]

Telomeres are a promising option to improve risk stratification of SCD in CAD patients given their ability to quantify accelerated biological ageing (which reflects the replicative potential of the cell and/or the cumulative lifelong burden of oxidative stress) distinct from an individual's chronological age. Keeping in mind the evidence demonstrating a relationship between telomere biology and cardiovascular disease and taking into account the mechanisms of telomere shortening described before; it was hypothesised that telomere shortening due

at the time of myocardial infarction results in poor myocardial repair process and predisposes patients to greater arrhythmic tendency.

To test this hypothesis, a retrospective case-control study was designed wherein the association between leukocyte telomere dynamics (mean telomere length, telomerase activity and load-of-short telomeres) and ventricular arrhythmias in ischaemic cardiomyopathy patients was examined.

3.2 Methods

This case-control observational study was carried out at St Bartholomew's hospital, William Harvey Research Institute and Vejle hospital, Denmark. Ethical approval was obtained from the local research ethics committee. All patients gave written informed consent and were recruited from the ICD clinic at St Bartholomew's Hospital. Investigators were blinded to clinical history and all telomere analysis was carried out blinded to clinical data.

3.2.1 Subjects

A total of 350 patients attending the ICD clinic at St Bartholomew's hospital were screened over one year (February 2011 – February 2012). Of these 140 met the inclusion criteria and 90 agreed to participate in the study.

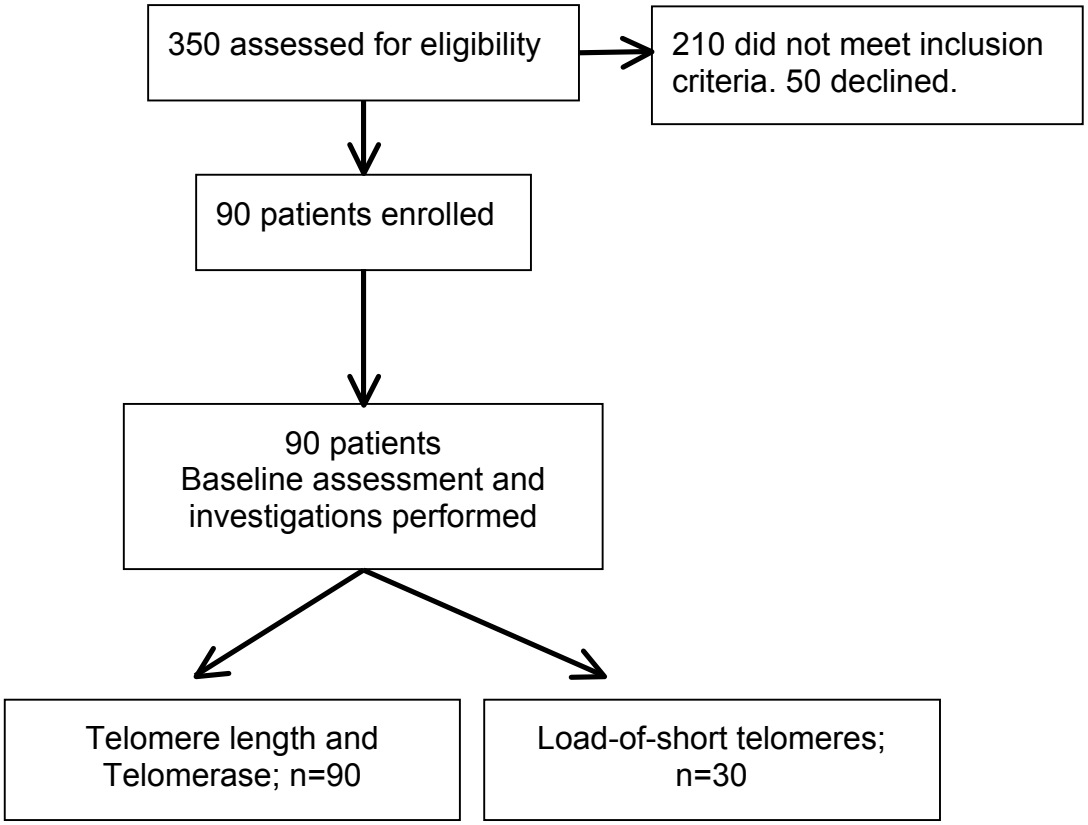

Figure 10: Number of patients recruited (Boxes show number of patients included)

Inclusion and exclusion criteria governing recruitment are described in detail in chapter 2. The major reasons for exclusion were non-ischaemic cardiomyopathy, secondary prevention ICDs and gout.

Of the 90 patients enrolled in the study, the cases comprised 35 patients who had primary prevention ICDs on a background of ischaemic cardiomyopathy and had received appropriate therapy from the ICD for potentially-fatal VA. The control cohort comprised of 55 patients with ischaemic cardiomyopathy who had primary prevention ICDs and had never received any therapy from their device. Diagnosis was verified by inspection of hospital records.

Mean telomere length and telomerase activity were measured on all of the cases and controls recruited. A randomly selected subset of male patients form the above cohort were recruited for the analysis of critically short

telomeres. A total of 30 patients were included, 15 in the cases and 15 in the control arm.

3.2.2 Measurements

Clinical

A standard questionnaire was filled in for all enrolled patients. Clinical details were obtained at recruitment by interviewing the patient and by examining their hospital records. The questionnaire recorded their medical history (to identify hypertension, diabetes, chronic renal failure, hypercholesterolemia, smoking status, family history of ischaemic heart disease and any other significant medical conditions), drug history, NYHA class, details of ICD implant, programming and previous arrhythmia history.

At recruitment, the patients also underwent a trans-thoracic echocardiogram to assess left ventricular function (LVEF measured by modified simpsons method) and detailed ICD interrogation to identify therapies delivered. A peripheral venous blood sample for general labs (including renal function, inflammatory markers - WCC and CRP, lipid profile), brain natriuretic peptide (BNP) and telomere biology in duplicate.

Phenotypic assessment is detailed in Chapter 2, section 2.3

Telomere biology analysis

Leukocytes were extracted and buffy coat aliquots prepared from whole blood within 30 min of venepuncture and genomic DNA extracted from them as described in chapter 2. Quantitative real time PCR analysis was used for analyses of mean telomere length, universal single telomere length analysis for quantifying critically short telomeres and telomere repeat amplification protocol assay for analysis of telomerase activity as detailed in the methods section in

chapter 2. All samples were analysed in duplicate and blinded to the clinical data.

3.2.3 Study End Points

The primary end point of the study was the difference in telomere dynamics (mean telomere length, telomerase activity, load-of-short telomeres) between patients subsequently experiencing appropriate ICD therapies for potentially fatal VA compared to those without any appropriate ICD therapies. ICD therapies were used as a surrogate for SCD and promoted differentiation between cases and controls. Potentially fatal VA was defined as sustained ventricular tachycardia at a rate > 170 bpm (and lasting at least 30 seconds) or ventricular fibrillation requiring device therapy. This was based on the programming criteria for primary prevention implants at our institution as explained in section 2.3.

Secondary end points included correlation between telomere dynamics and LVEF, eGFR and serum BNP.

3.2.4 Statistical Analyses

Sample size was determined by designing the study to have 90% power to detect a significant difference in telomere dynamics between the potentially fatal and no potentially fatal VA groups at a type 1 error of 5%. A significant difference in the primary outcome measure was defined as 300bp mean telomere length difference between the cases and controls. This was based on published literature and a strong correlation observed between telomere length assessment by TRF analyses and qPCR in previous experiments performed by our group. For telomerase activity, the sample size was based on pilot data derived from initial analyses on 20 patients. Telomerase studies, to date, have used an average sample size of 20 patients. Not enough evidence was

available from published cardiovascular literature to substantiate power calculations for this study. Hence, the average sample size (n = 20) used in published telomerase studies was used to obtain initial data, the results of which formed the basis of power calculations for the telomerase cohort. It was necessary to transform the telomerase activity to Log (Telomere + 0.0001). The calculations assume a difference of 0.8 in the two means with a pooled standard deviation of 1.3658. 15 patients in the cases cohort and 15 in the controls were required for the load-of-short telomeres based on the expected difference between groups of 0.2 with a standard deviation of 0.2.

Continuous variables are reported as mean ± standard deviation, or median (range) if not normally distributed. Characteristics of cases and controls were compared using chi-squared test for categorical variables and unpaired t-test for continuous variables if normally distributed or Mann-Whitney U test if not normally distributed. The effects of age, gender, WCC, genotype and other individual cardiovascular risk factors on mean telomere length, telomerase activity and load-of-short telomeres were assessed using regression models. The above telomere dynamics, as a risk factor for appropriate ICD therapy for potential fatal VA (surrogate for SCD), were assessed using logistic regression. All analyses was carried out using SAS VERSION 9.3 stats software.

3.3 Results

3.3.1 Demographics

The baseline demographic characteristics of cases and controls are shown in Table 7.

Mean Telomere Length & Telomerase	Cases	Controls	p-value
Number of patients	35	55	
Age(yrs), mean±SD	69.3±7.6	71.6±8.8	0.19
White cell count (109/L), mean±SD	7.7±1.7	7.46±1.7	0.43
Male gender,n(%)	32(91.4)	48(87.3)	0.78
Diabetes,n(%)	7 (20)	18 (32.7)	0.28
Hypertension,n(%)	14 (40)	25 (45.4)	0.77
Time from MI to ICD implant, months;mean±SE	106.8±14.5	89.5±12.0	0.36
Time from MI to recruitment, months;mean±SE	138.51±12.98	99.27±22.41	0.19
Time from ICD implant to recruitment, months;mean±SE	46.5±4.55	38.1±4.11	0.18
LVEF,%;mean±SD	26.73±7.2	27.24±7.6	0.75
eGFR, mean±SD	63.94±21.8	60.59±20.22	0.49
NYHA GradeIII, n(%)	33 (94.28)	50 (90.91)	0.62
Load-of-short telomeres			
Number of patients	15	15	
Age, mean±SD	72.2±7.4	71.2±9.6	0.76
White cell count (109/L), mean±SD	8.03±1.4	7.75±1.6	0.64
Male gender,n(%)	15 (100)	15 (100)	1
Diabetes,n(%)	3 (20)	3 (20)	1
Hypertension,n(%)	3 (20)	4 (26.6)	0.67
Time from MI to ICD implant, months;mean±SD	130.87±22.28	133.64±21.78	0.93
Time from MI to recruitment, months;mean±SD	168.9±17.54	141±20.67	0.31
Time from ICD implant to recruitment, months;mean±SE	48.3±7.64	38.3±8.03	0.4
LVEF,%;mean± SD	25.3±6.4	26.9±7.9	0.55
eGFR, mean±SD	56.7±17.9	54.1±17.3	0.69
NYHA GradeIII, n(%)	12 (80)	13(86.6)	0.81

Table 7: Baseline demographics of cases and controls included in the study

[Results shown as mean±standard deviation or number of patients and % of total group. LVEF: Left Ventricular Ejection Fraction, eGFR: Glomerular Filteration Rate, NYHA: New York Heart Association classification]

All recruited subjects were Caucasians. The cases and controls were well matched for age and gender. The mean age at recruitment was 69.3 ± 7.6 years for cases and 71.6 ± 8.8 years for controls. There was no significant difference in cardiovascular risk factors between the two groups. The vast

majority of patients had NYHA grade III amongst cases and controls and the LVEF was less than 35% across the entire cohort in keeping with the inclusion criteria. The mean WCC in cases was 7.7±1.7 and controls 7.46±1.7, indicating no significant difference in their inflammatory status. At recruitment, the time since ICD implant and time from MI to ICD implant was similar in cases and controls (46.5±4.55, 38.1±4.11, p = 0.18 and 106.8±14.5, 89.5±12.0, p = 0.36 months in cases and controls respectively).

The subset of patients for the analysis of critically short telomeres exhibited a similar demographic profile. All subjects were men and cases and controls were well matched for age. There were no significant differences in cardiovascular risk factors, renal function, WCC, LVEF, NYHA class and time since ICD implant between cases and controls as shown in table 7.

3.3.2 Correlation of Clinical Parameters with ICD Therapy

A univariate analysis was performed to assess the correlation of clinical parameters with ICD therapy. There was no significant correlation between ICD therapy and gender (OR 0.63, CI 0.151 – 2.618, p-value 0.524), diabetes (OR 0.50, CI 0.184 – 1.380, p-value 0.18), hypertension (OR 0.93, CI 0.39 – 2.232, p-value 0.87), WCC (OR 1.11, CI 0.865 – 1.425, p-value 0.41), LDL (OR 1.822, CI 0.913 – 3.634, p-value 0.09), eGFR (OR 1.01, CI 0.989 – 1.033, p-value 0.34) and LVEF (OR 1, CI 0.945 – 1.059, p-value 0.98). Although correlation with gender was computed, this was probably skewed by the fact that the number of females in both groups was very small. The absence of a significant correlation with LVEF can be explained by the narrow range of LVEF in our patient cohort based on inclusion criteria. The only significant correlation seen was with age of the patient (OR 0.93, CI 0.184 – 0.986, p-value 0.01). The correlation analysis of ICD therapy with clinical parameters is shown in Table 8.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Gender	0.63	0.151 – 2.618	0.524
Age	0.93	0.887 – 0.986	0.01
Diabetes	0.50	0.184 – 1.380	0.18
Hypertension	0.93	0.39 – 2.232	0.87
WCC	1.11	0.865 – 1.425	0.41
LDL	1.822	0.913 – 3.634	0.09
BNP	0.99	0.985 – 1.012	0.82
eGFR	1.01	0.989 – 1.033	0.34
LVEF	1.00	0.945 – 1.059	0.98

Table 8: Univariate correlation analysis of ICD therapy with clinical parameters

[WCC: White Cell Count, LDL: Low Density Lipoprotein, BNP: Brain Natriuretic Peptide, GFR: Glomerular Filteration Rate, LVEF: Left Ventricular Ejection Fraction]

3.3.3 Mean Telomere Length

The mean white cell telomere length as a function of age is shown in Figure 11 for both cases and controls. As expected, the mean telomere length ratio decreased steadily with age at a mean yearly rate of 0.0063 ± 0.0032, with evidence of inter-individual variation in telomere length at any given

chronological age- Figure11. This rate of decline in telomere length with age was similar to that noted in other studies. [98,158]

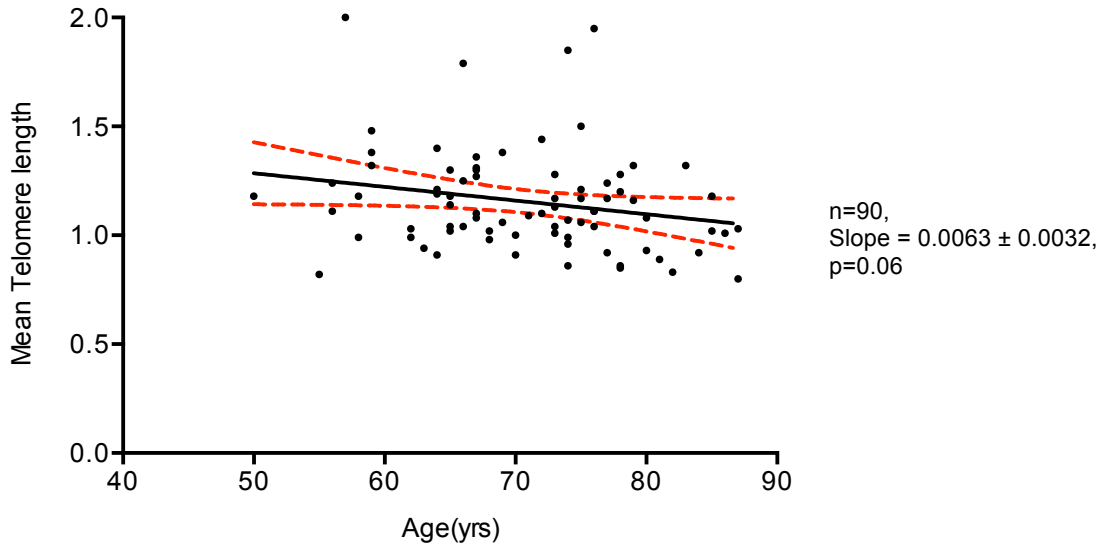

Figure 11: Correlation of mean telomere length with age

This correlation between mean telomere length and age nearly reached significance (p=0.06). The very narrow interquartile age range (62 – 75 yrs) in our cohort may account for the weak correlation (despite having a similar rate of decline) observed when compared to other telomere studies.

A univariate analysis did not show any significant correlation between mean telomere length and ICD therapy as shown in the table 9 (OR 1.48, CI 0.253 – 8.762, p-value 0.66).

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Mean telomere length	1.488	0.253 – 8.762	0.66

Table 9: Univariate correlation analysis of ICD therapy and mean telomere length

This correlation was further weakened when telomere length was adjusted for age, WCC and gender in a multivariate analysis as shown in table 10.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Mean telomere length	0.858	0.108 – 6.833	0.88
Age	0.95	0.896 – 1.007	0.06
Gender	0.496	0.048 – 5.107	0.55
WCC	1.056	0.804 – 1.386	0.69

Table 10: Multivariate correlation analysis of ICD therapy and mean telomere length

There was no significant difference in the mean telomere length between cases (fatal VA) and controls (no-fatal VA), p=0.66, as shown in figure 12 below. Also, age, gender and WCC adjusted mean telomere length did not have a significant correlation with incidence of ICD therapy (OR 0.858, CI 0.108 – 6.833, p-value 0.88).

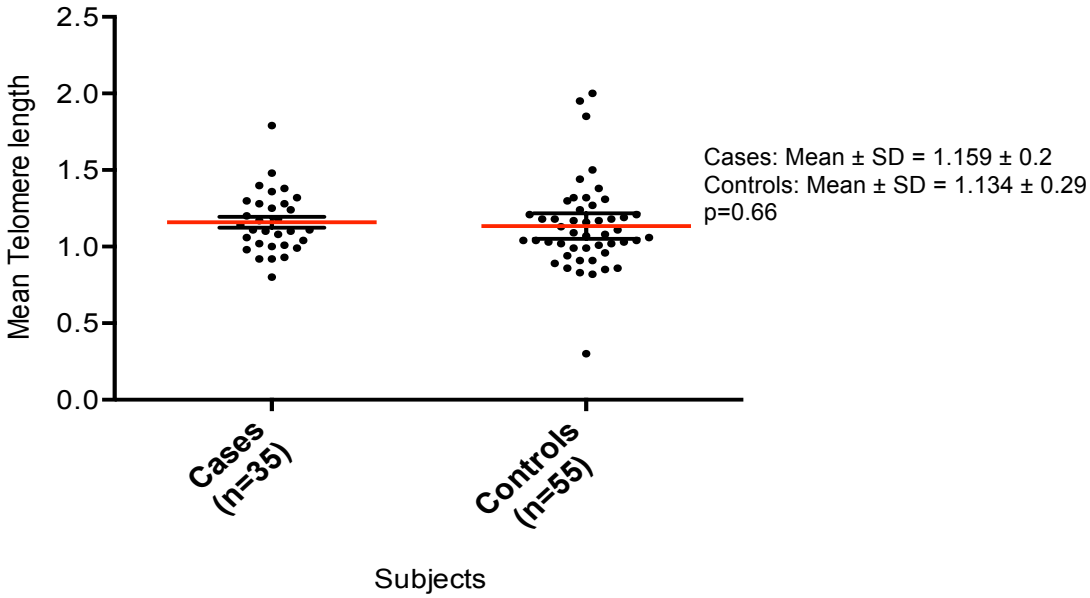

Figure 12: Mean telomere length (with 95% confidence interval) in cases and controls

3.3.4 Telomerase Activity

The leukocyte telomerase activity (n = 90) as a function of age is shown in the figure 13 for both cases and controls.

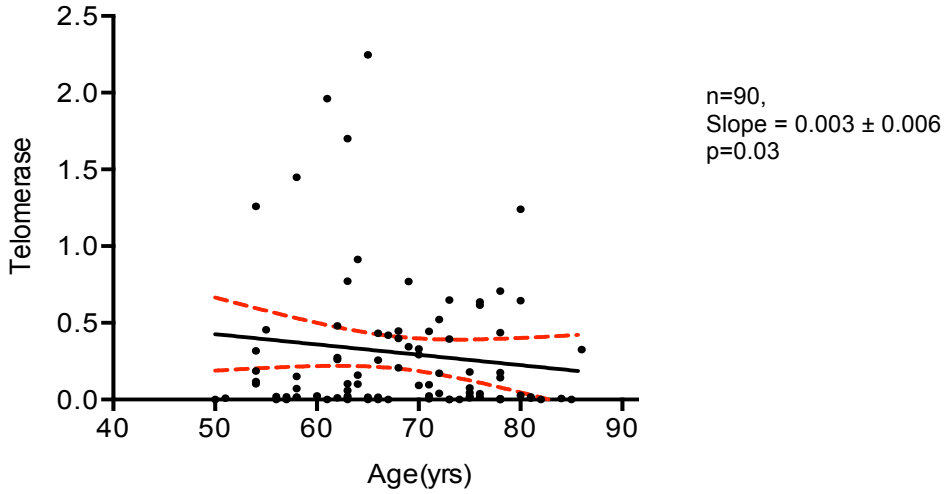

Figure 13: Correlation of telomerase activity with age

Leukocyte telomerase activity was seen to decrease significantly with increasing chronological age (n = 90, p = 0.03) – Figure 13. This has not been consistently reported in the small number of clinical telomerase studies conducted so far.

A univariate analysis showed a highly significant correlation between telomerase activity and ICD therapy as shown in table 11 (OR 4.8, CI 1.46 – 15.78, p-value 0.009).

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity	4.8	1.46 – 15.78	0.009

Table 11: Univariate correlation analysis of ICD therapy and telomerase activity

This correlation still remained significant when telomerase activity was adjusted for age, gender and WCC as shown in table 12.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity	5.302	1.293 – 21.742	0.02
Age	0.938	0.885 – 0.993	0.03
Gender	0.845	0.138 – 5.158	0.85
WCC	0.996	0.751 – 1.322	0.98

Table 12: Multivariate correlation analysis of ICD therapy and telomerase activity

There was a significant difference in the telomerase activity between cases (fatal VA) and controls (no fatal VA) with the telomerase activity being much higher in cases (0.50 versus 0.18, p = 0.009) as shown in the figure 14 below. Also, the age, gender and WCC adjusted telomerase activity had a significant correlation with incidence of ICD therapy (OR 5.302, CI 1.293 – 21.742, p-value 0.02).

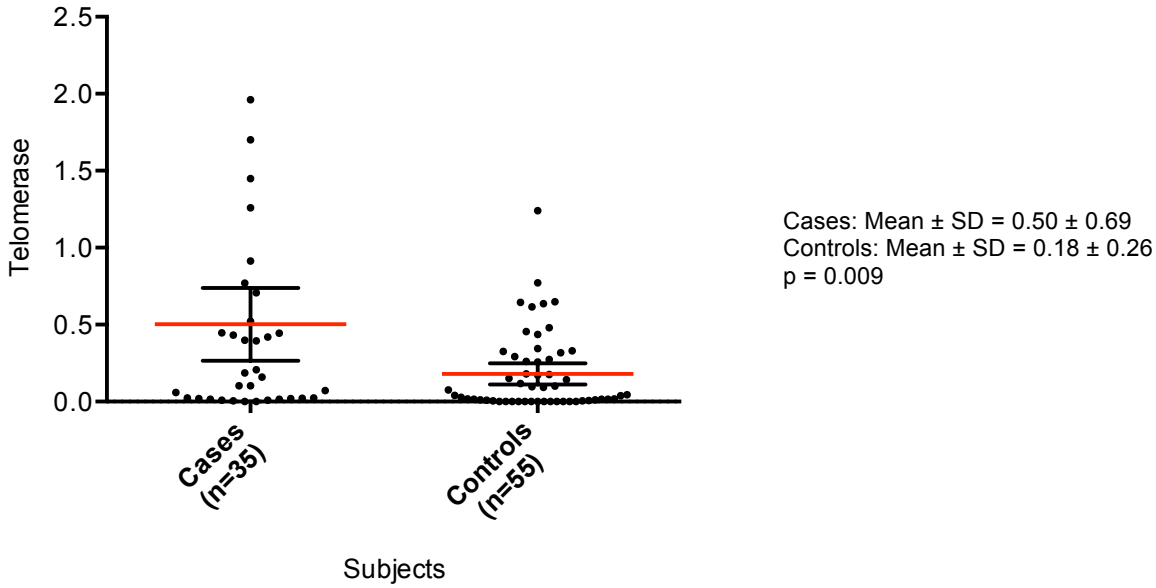

Figure 14: Telomerase activity (with 95% confidence interval) in cases and controls

3.3.5 Critically Short Telomeres

The load-of-short telomeres was calculated based on the number of bands below 1500 bp. The threshold of 1500 bp was chosen as the cut-off for critically short telomeres largely based on previously published literature [152,179] and also similar findings replicated in this study. The exact threshold at which telomeres become critically short is difficult to ascertain and may perhaps even vary between cell types. In all studies carried out with U-STELA to date, almost identical results were obtained with thresholds set at 1000bp, 1500 bp and

2000 bp. However, bands below 1000 bp were infrequent and hence a single potential questionable band could impact the results. At 2000 bp, a consistent increase in the clustering of bands was seen which might be a consequence of gradual replicative shortening. Bands below 1500 bp were hence thought to best represent telomeres derived from stochastic shortening and have been used as a threshold in published studies to date. This, however, is based on a limited number of published studies and a larger evidence base is awaited.

The U-STELA gels from which the load-of-short telomeres were derived are shown in Appendix A, alongwith the resultant bands quantified using Vision Works LS Acquisition and Analysis software. An example of the U-STELA gel with markers is shown in Figure 15 and the resultant quantified bands in Table 13.

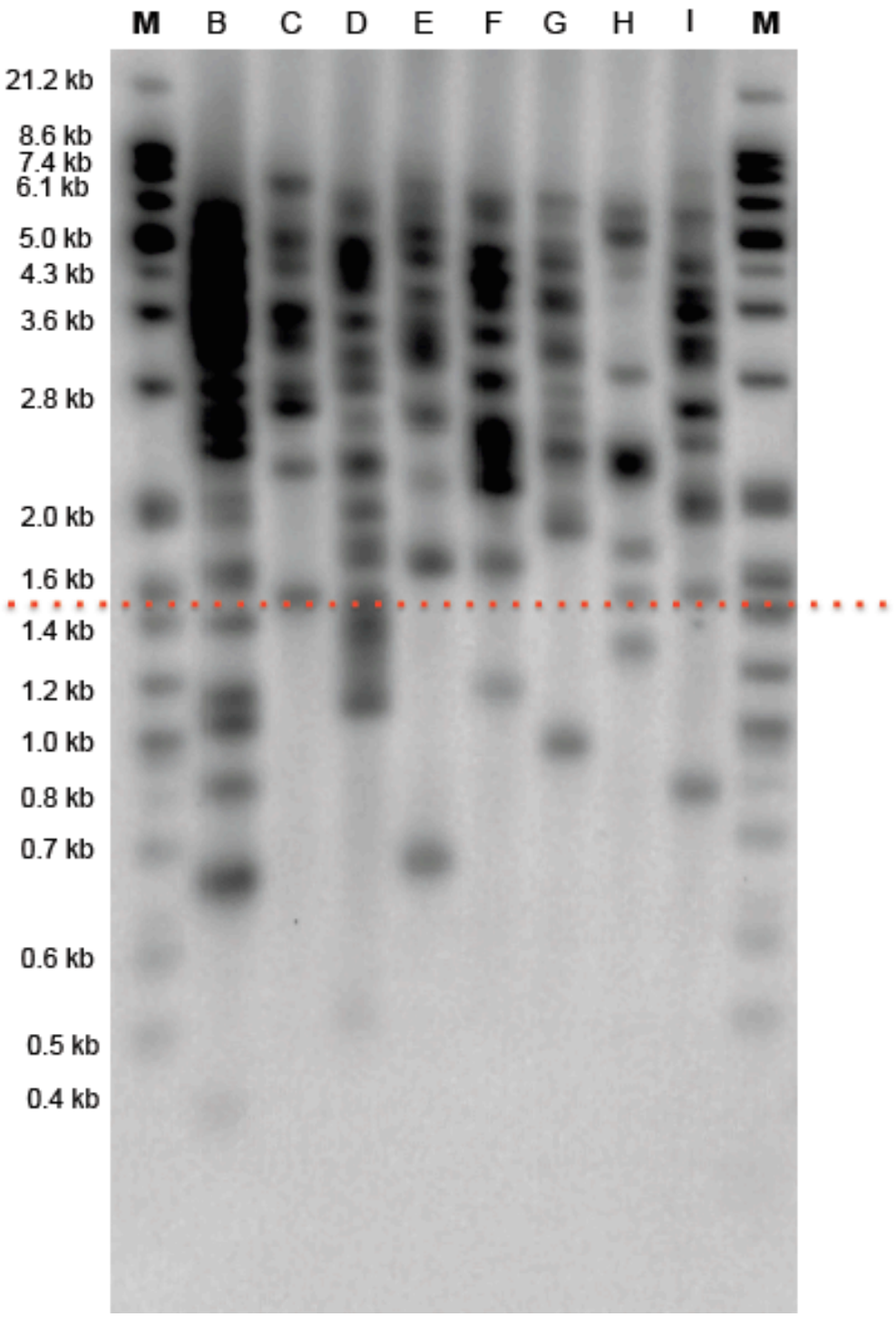

Figure 15: U-STELA gel with markers

Band Gel1_1	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2335.619	2640.817	2540.360	2573.414	2475.521	2763.049	2246.772	2606.898	21226
2	8576	2039.161	2217.914	2232.297	2133.545	2133.545	2556.834	1760.335	2396.794	8576
3	7427	1936.900	1540.748	1961.898	1694.401	1694.401	2350.765	1559.893	2000	7427
4	6106	1630.937		1760.335	695.723	1164	1875.976	1341.609	1540.748	6106
5	5000	1451.207		1477.508			957.914		849.003	5000
6	4268	1138.895		1416.865						4268
7	3630	1059.057		1122.459						3630
8	2799	849.002		387.603						2799
9	2000	654.593								2000
10	1900	280.425								1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 13: Telomere bands quantified from U-STELA gel

The number of bands below 1500 bp were counted and calibrated based on the initial PCR template concentration. These were then presented as the number of telomeres below 1500 bp per genome equivalent of template DNA used and comparison made between cases and controls. All analysed gels with quantified telomere bands are shown in Appendix A.

The load-of-short telomeres as a function of age are shown in the figure below (Figure16) for both cases and controls. There was no correlation between the load-of-short telomeres and age ($p = 0.57$).

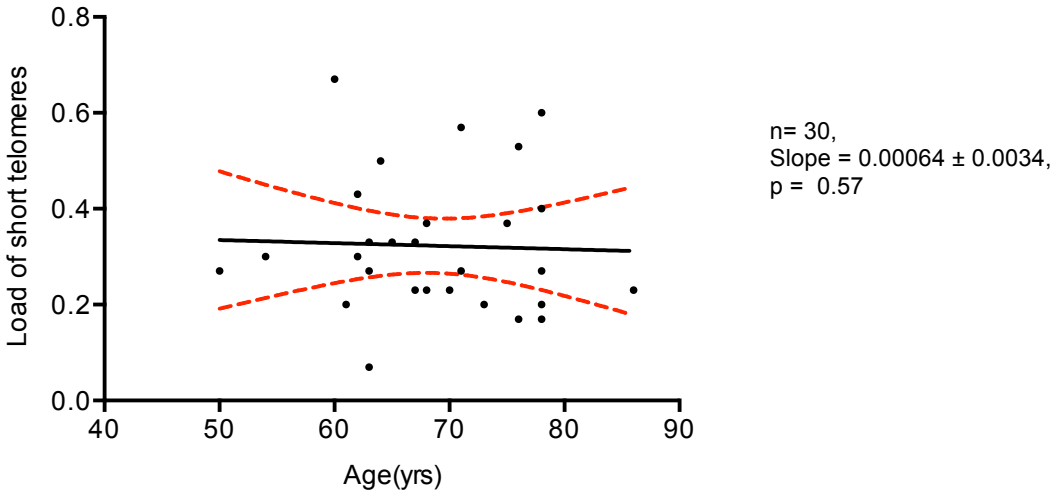

Figure 16: Correlation of load-of-short telomeres with age

A univariate analysis showed a significant correlation between load-of-short telomeres and ICD therapy in ischaemic cardiomyopathy patients (OR 3.478, CI 1.202 – 10.064, p-value 0.02) – table 14.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Load-of-short telomeres	3.478	1.202 – 10.064	0.02

Table 14: Univariate correlation analysis of ICD therapy and load-of-short telomeres

This correlation remained significant despite adjustment for other parameters including age and WCC in multivariate analysis as shown in table 15 below.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Load-of-short telomeres	4.560	1.185 – 17.541	0.02
Age	0.963	0.846 – 1.097	0.57
WCC	0.986	0.458 – 2.123	0.97

Table 15: Multivariate correlation analysis of ICD therapy and load-of-short telomeres

There was a significant difference in the load-of-short telomeres between cases (fatal VA) and controls (no fatal VA) with the load-of-short telomeres being much higher in cases (n = 30, p = 0.02) as shown in the figure below. Also, the age and WCC adjusted load-of-short telomeres had a significant correlation with incidence of ICD therapy (OR 4.560, CI 1.185 – 17.541, p-value 0.02) – Figure 17.

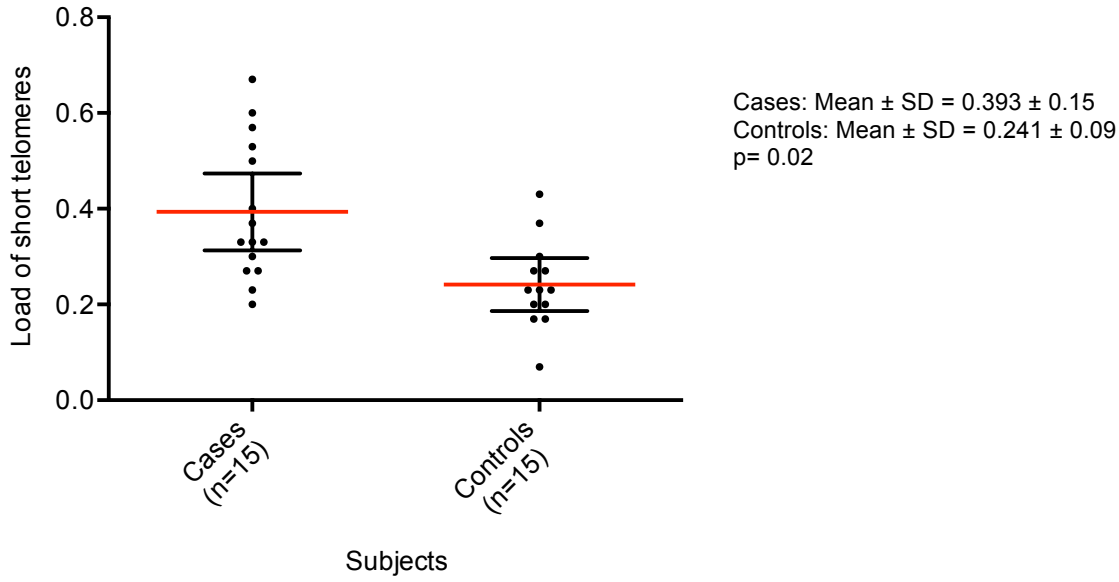

Figure 17: Load of short telomeres (with 95% confidence interval) in cases and controls

We also looked at mean telomere lengths (in cases and controls) in this subset of 30 patients who had critically short telomeres analysed. In keeping with the earlier analyses of mean telomere length (n = 90), there was no significant difference between cases and controls – Figure 18.

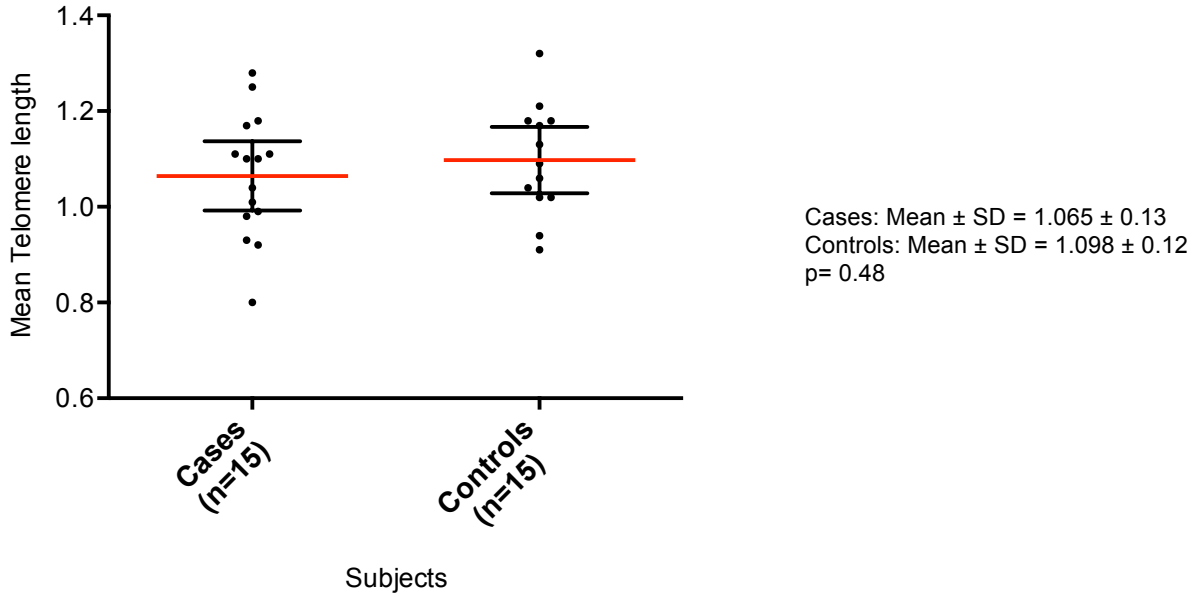

Figure 18: Mean telomere length (with 95% confidence intervals) in cases and controls in the cohort analysed for load analyses (n = 30)

3.3.6 Correlation of Telomere Dynamics with Time to Therapy from ICD implant

A multivariate analysis was performed to assess the correlation between age and gender adjusted mean telomere length and telomerase activity with time to ICD therapy from the date of ICD implant. This is shown in table 16.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity	1.372	0.762 – 2.471	0.292
Mean telomere length	1.430	0.100 – 20.441	0.79
Age	0.954	0.890 – 1.023	0.19
Gender	0.626	0.075 – 5.215	0.66

Table 16: Multivariate correlation analysis of telomere dynamics with time to therapy from ICD implant

There was no significant correlation between time to ICD therapy with mean telomere length (OR 1.43, CI 0.10 – 20.44, p-value 0.79) and telomerase activity (OR 1.372, CI 0.762 – 2.471, p-value 0.292) from the time since ICD implant.

3.3.7 Sensitivity and specificity of telomere dynamics in predicting VA

ROC analysis was performed to determine the sensitivity and specificity of telomere dynamics to determine the incidence of VA in this patient cohort. The area under the curves (AUC) data indicated that the load of short telomeres and telomerase activity were better at determining the incidence of cardiac arrhythmia in ischaemic cardiomyopathy patients than LVEF (AUC 0.82, 0.66 and 0.47 for load, telomerase and LVEF respectively) – Figure19.

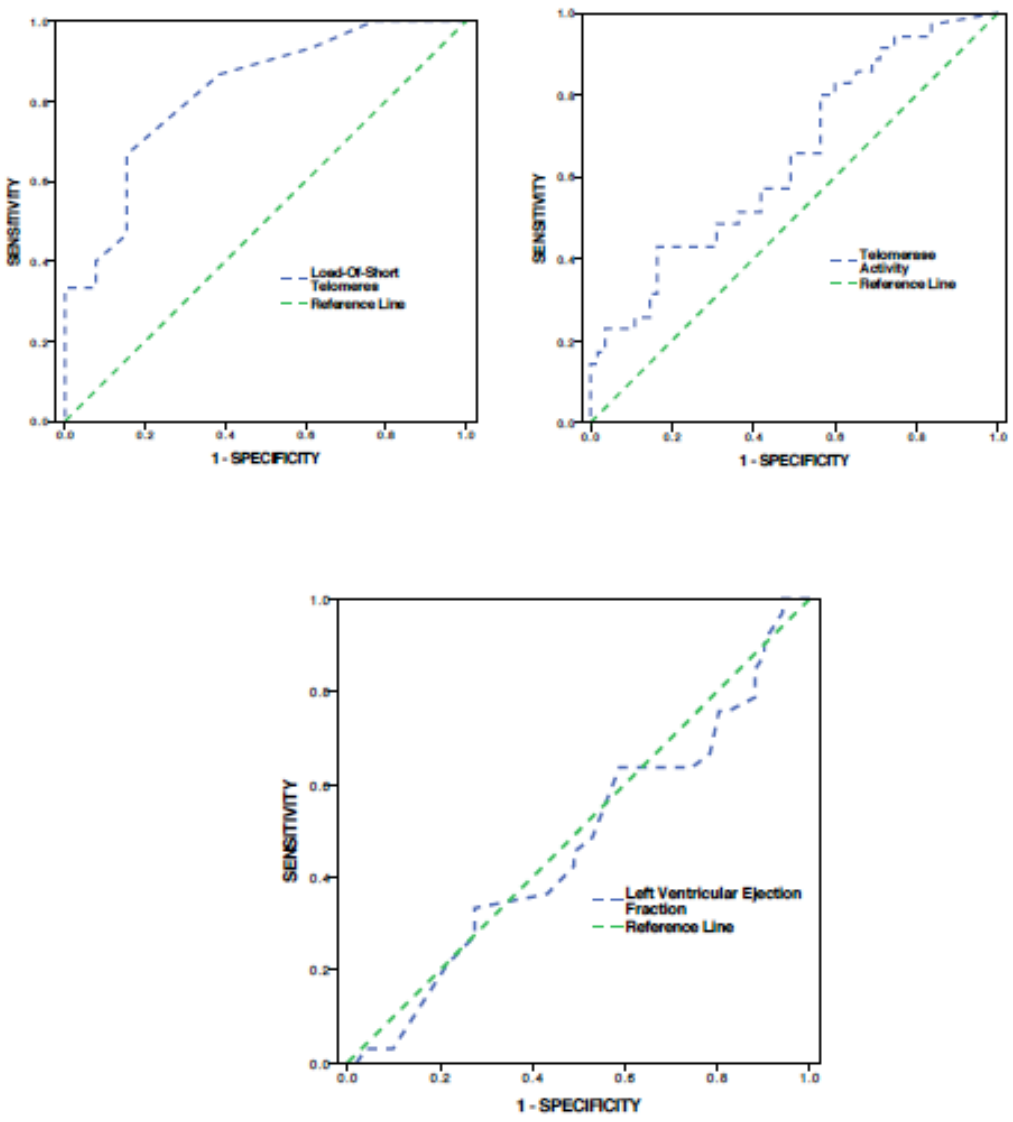

Figure 19: ROC analysis of telomere dynamics and LVEF with ICD therapy

3.3.8 Correlation of Telomere Dynamics with LVEF, eGFR and BNP

Investigating the secondary end points of the study, it was noted that there was no significant correlation between telomerase activity and eGFR (p value 0.11), BNP (p value 0.29) and LVEF (p value 0.55). Also, the load of short telomeres did not correlate significantly with eGFR (p value 0.76), BNP (p value 0.18) and LVEF (p value 0.42).

Similarly, there was no significant correlation between mean telomere length and eGFR (p value 0.64) and BNP (p value 0.45). However, a significant correlation was noted between the mean telomere length and LVEF (p-value 0.04) in this group of ischaemic cardiomyopathy patients. This was despite the very narrow range of LVEF 10% - 35% present in the study cohort. The correlation plot between mean telomere length and LVEF for both cases and controls is shown in figure 20 below.

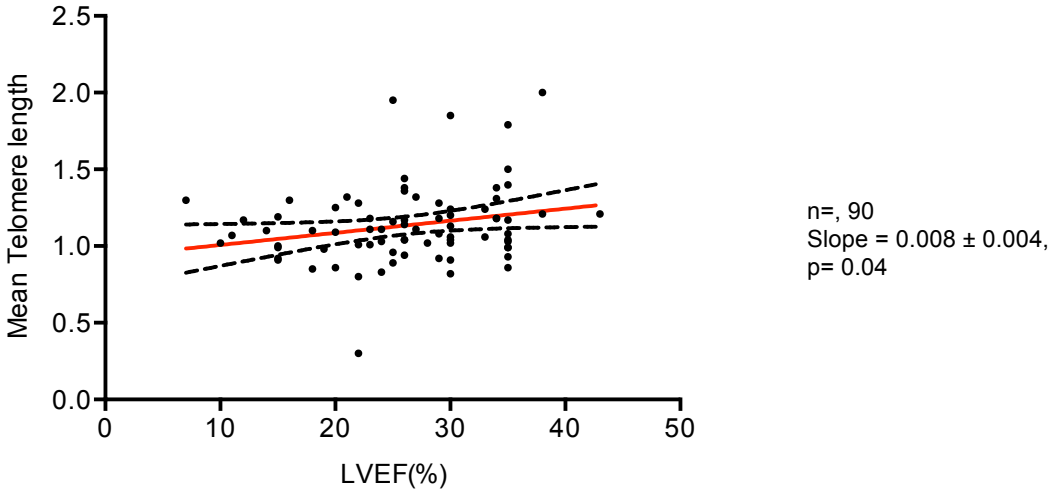

Figure 20: Correlation of telomere length and LVEF in cases and controls

3.4. Discussion

3.4.1 Overview of study results

The principal finding of this study is that the load of critically short telomeres is greater and telomerase activity higher in white blood cells obtained from peripheral blood of patients with ischaemic cardiomyopathy who have received ICD therapy for potentially fatal cardiac arrhythmia compared to patients with no VAs. A similar observation was not found in the mean telomere length measurement.

The entire cohort comprised of individuals with severely impaired left ventricular function post MI and had an ICD fitted for prevention of sudden death from cardiac arrhythmia. All recruited patients were Caucasians as ethnic variation can confound telomere biology results. [97,101] Both cases and controls had a similar burden of CAD, cardiovascular risk factors, time since ICD implant, inflammatory status and were of a similar age group and hence negating the effect of potential confounding factors on telomere biology assessment.

Despite LVEF being the defining criteria for ICD implants post MI, we did not find a significant association between LVEF and ICD therapy. This is understandable in this patient cohort due to a very narrow range of LVEF present and also due to the exclusion of any patients with normal left ventricular function. Of the various clinical parameters studied it was only the age of the patient that had a significant association with ICD therapy. This was adjusted for in all further analysis.

The leukocyte telomerase activity increased as a function of increasing chronological age. This correlation was significant despite a narrow age range present in this study cohort. This could reflect a protective response to the

decreasing telomere length with increasing age. Moreover, the telomerase activity was higher in the cases when compared to controls and exhibited a significant correlation with ICD therapy, even after adjustment for age, WCC and gender. This finding becomes more important prognostically, as there was no significant association between telomerase activity and time to ICD therapy (from implant) or the number of therapies received. This implies that telomerase activity may have a causal role in determining ICD therapy in ischaemic cardiomyopathy patients.

Likewise, the load of critically short telomeres was greater in the cases when compared to the controls and correlated significantly with ICD therapy in a multivariate regression analysis. Importantly this correlation became more significant with decreasing telomere threshold from 2000bp to 1000bp and hence strengthening the validity of the observed association.

As seen in previous studies, we found that mean telomere length decreased with increasing chronological age. However, this correlation was not as significant as that seen in some other studies, [96,97,158] which is largely down to the very narrow interquartile age range (62 - 75 yrs) and absence of significant numbers of younger age group in our patient population. Moreover, in a heterogenous human population, demonstration of a significant age-dependent attrition in mean telomere length is only possible when the age distribution is sufficiently wide. This is due to the wide scatter of mean telomere length among individuals of the same age and also the slow age dependent attrition of telomere length. Contrary to the other telomere markers studied, the mean telomere length did not vary much between cases and controls and did not have any significant correlation with ICD therapy. This suggests that the

incidence of cardiac arrhythmia necessitating ICD therapy was closely associated with the shortest telomere rather than the mean telomere length.

There was no significant association between the telomere biology markers studied and several other demographic characteristics, cardiovascular risk factors and markers of inflammation in this study. These factors did not confound or explain the association (or lack of) seen between telomere biology markers and ICD therapy. There was no independent effect of gender on telomere biology markers. This is contrary to what has been found in other studies that have shown females to have shorter telomeres than men.[163,180,181] The lack of association with gender might be due to the small number of females present in the study. The absence of association between telomere biology and inflammatory markers studied, does not exclude the possibility that the chronic inflammation and oxidative stress is an important factor contributing to the observed difference in telomere biology markers between cases and controls. Even if these markers provided a reliable assessment of inflammation, it is unlikely that a single point measurement of these variables would accurately reflect the cumulative burden of inflammation and oxidative stress that is likely to have an impact on telomere biology. Another possibility is that these markers may be more relevant in the pre-clinical phase of the disease as inflammation and oxidative stress promote atherogenesis. However, once CAD is established other factors (genetic and environmental) may have a greater role in determining biological ageing and hence telomere attrition. [182]

ROC analysis indicated that telomere markers (load of short telomeres and telomerase activity) were better at determining the incidence of VA than LVEF alone in ischaemic cardiomyopathy patients. However, it has to be considered that all patients had a LVEF ≤ 35% and these results may vary if a

wider range (including normal) of LVEF is used. This was not possible within the realms of this study.

Amongst the secondary end points studied, there was a significant correlation between mean telomere length and LVEF. The mean telomere length decreased with a worsening left ventricular function. This was despite the narrow range of LVEF (10 – 35%) present in this patient cohort. This significant association between a biological marker of ageing and a clinically relevant marker of cardiac function, indicates that the cumulative biological stress effects myocardial function in patients with CAD. Whether this association is independent of the presence of CAD, is difficult to establish, given the current design of the study.

3.4.2 Possible reasons for the observed differences in telomere dynamics between cases and controls and the mechanisms involved

In this study the load of short telomeres was greater and telomerase activity higher in cases than controls whereas the mean telomere length was similar across the two groups. This difference (or lack of) in the telomere biology markers between cases and controls could be due to a number of different reasons.

<u>The observed difference could be an artefact.</u>

It is highly unlikely that the observed difference is an artefact as the strength of correlation between telomere biology markers and ICD therapy is very strong. Univariate correlation analysis of ICD therapy with telomerase activity was significant at a p-value of 0.009, and with load of short telomeres at a p-value of 0.02. This correlation remained significant in multivariate regression analysis both for telomerase activity (p-value 0.02) and load of short telomeres (p value

0.02). Moreover, for the load of short telomeres, it became more significant as lower thresholds of telomere length were used (at 1500 bp, p-value 0.02; at 1000 bp, p-value 0.001).

Also, it is worthwhile to note that the observed difference was not present in all markers of telomere biology. The mean telomere length was not significantly different between cases and controls.

All recruited patients inherited a similar profile of telomere biology markers. Different rates of attrition due to environmental factors resulted in the observed difference.

If we are to assume that all subjects were born with the same telomere length and the same level of telomerase activity, then the observed difference would be a result of different rates of telomere attrition in cases and controls secondary to acquired external factors. In this study cohort, all patients had a prior MI and we hypothesised that telomere attrition at the time of MI results in poor myocardial repair process and predisposes patients to greater arrhythmic tendency. Hence, leukocyte telomere biology markers will be different in ischaemic cardiomyopathy patients with and without potentially fatal VA and these would correlate with the incidence of VA (ICD therapy).

Atherosclerosis and CAD were present in both cases and controls, whereby they were exposed to both inflammatory burden and oxidative stress, which form an integral part of this disease process. Both inflammation and oxidative stress (reactive oxygen species) are central to the pathobiology of age related cardiovascular disorders and biological ageing. Inflammation is associated with increased cell proliferation, accelerating the attrition rate due to end replication problem (mean telomere length). Oxidative stress results in the formation of single strand breaks, preferentially at the telomeres, resulting in

stochastic telomeric shortening (load of short telomeres) and causing an accelerated telomeric shortening at the next cell division (mean telomere length).

Inflammation has a central role in all stages of the atherosclerotic process from initiation and evolution of atheroma to decisively contributing to the precipitation of acute thrombotic complications of atheroma including MI. [183] This may have a direct effect on telomere attrition due to increased cell turnover secondary to inflammation and hence contributing to the replicative senescence of telomeres. Moreover, cellular senescence has been shown to be a major feature of atherosclerotic plaques, [184,185] and the finding that interference with telomere function in coronary endothelial cells in vitro causes expression of molecules implicated in atherogenesis, [186] suggests that shorter telomeres could contribute directly to the pathophysiology of atherosclerosis.

Oxidative stress, wherein production of free radicals – reactive oxygen species (ROS) – exceeds the cellular antioxidant capacity, has also been shown to play a major role in atherosclerosis and cellular senescence. This is by virtue of its role in regulating cellular proliferation, differentiation and cellular death [187,188] and also its role in activation of the apoptosis and the resultant impact on the ageing process. [189] This is exemplified in the widely accepted “free radical theory of ageing” proposed by Harman. [190] There is an exponential increase in oxidative damage and reduction in antioxidant defenses with increasing chronological age and hence the resultant cellular senescence and premature biological ageing secondary to increased oxidative stress. [190,191] A major factor mediating this process is thought to be stochastic shortening of telomeres and compromised telomeric integrity. [130,192]

Testing the hypothesis in this study, no striking association was seen between the mean telomere length and cardiac arrhythmia occurrence in ischaemic cardiomyopathy patients. This represents an important contrast to the studies that have shown a relationship between shorter mean telomere length and cardiovascular disease. [95-98,193] However, the present study compares cases and controls with established CAD and left ventricular impairment, in contrast to the widely distinct phenotypes evaluated in previously published studies. Hence, it might not be possible to detect a subtle difference in mean telomere length between cases and controls due the inability of a single time point measurement to reflect a gradual linear decline in mean telomere length in two closely related groups. However, this “small” difference in telomere attrition was clearly reflected in the load of short telomeres as the number of telomeres below 1500bp was significantly lower in cases compared to controls.

Traditionally, the vast majority of studies evaluating telomere biology in cardiovascular disease have looked at mean telomere length in leukocytes. This represents impact of replicative senescence on telomere attrition and was described by Olovnikov as the “end replication problem”. [194] The enzyme telomerase is capable of extending telomeres but is generally suppressed in somatic cells. In many cells senescence and cell death occurs when mean telomere length reaches a critical value. Thus, mean telomere length provides a marker for biological age, with shorter telomeres indicating an increased biological age. However, this mechanism of telomere attrition and biological ageing was not well depicted in our study population, as mean telomere length did not have a significant association with cardiac arrhythmia. Other mechanisms of telomere attrition may be more relevant here, as reflected in the

load of short telomeres, and the widely studied replicative senescence in cardiovascular ageing might not be enough to explain the disease process.

The causal link between the gradual decrease in mean telomere length and cellular senescence has been questioned by a number of observations.[152] Firstly, senescence induction has been shown to be bimodal with an increasing proportion of cells entering senescence at late passages, [195] while mean telomere length seems to shorten in an almost linear fashion. [196-199] Secondly, in tissue cultures senescence was found to be induced with mean telomere length much longer than what would be expected to trigger the senescence mechanisms. As a result of these findings, it has been suggested that telomere associated senescence may not depend solely on a global decrease in mean telomere length and instead be triggered by a single, or a group of few, critically short telomeres within the cell. [200-202]

The formation of these critically short telomeres cannot be fully explained by replication-driven telomere shortening alone. These are likely to have been formed by a sudden loss of a large telomere fraction from a particular telomere. The proposed mechanisms for this stochastic shortening include oxidative damage inflicting single strand breaks in telomeres, [203-205] replication slippage and unequal sister chromatid exchange [206,207] and recombination causing circularization and thus deletion of distal repeats. [208-211] Vast majority of evidence so far suggests that oxidative stress is the most clinically relevant parameter leading to formation of critically short telomeres. Various observations have shown free oxygen radicals to be a major cause of telomere shortening in human fibroblasts in vitro: (1) telomeres in non-proliferating cells accumulate single strand breaks because of a specific deficiency in base excision repair [212,213] (2) these breaks result in telomere

shortening during DNA replication, probably by transiently stalling the replication fork [196,198] (3) oxidative stress increases the telomere shortening rate by up to one order of magnitude [205,212,214] (4) diminution of oxidative stress slows down the rate of telomere shortening and decelerates replicative senescence [215] (5) the telomere shortening rate correlates with the cell strain-specific anti-oxidative capacity. [216]

Based on the above observations, it is largely believed that telomere attrition is a combination of gradual linear shortening due to the end replication problem reflecting the number of cell divisions; superimposed by a more stochastic mechanism causing a sudden shortening of a single telomere which has the potential to trigger an early senescence response as cell proliferation proceeds. Oxidative stress augments telomere attrition per replication and the inflammation increases cellular turnover, which would further increase telomere shortening.

In the present study, critically short telomeres (likely to have been generated due to oxidative stress during index MI) appear to be the predominant mechanism resulting in premature senescence and arrhythmia prediction. This is suggested by our findings, which show that the load of short telomeres had a significant association with ICD therapy despite a similar mean telomere length across cases and controls. This postulate is further supported by a recent study performed by our group (Dr Bendix, Vejle hospital, Denmark), wherein a strong correlation was established between senescence and oxidative damage by treating human mesenchymal stem cells - hMSCs (primary and hTERT immortalized) with sub-lethal doses of hydrogen-peroxide which induces stochastic telomeric shortening. After exposure to acute oxidative stress a positive correlation was found between load of short

telomeres and the percentage of senescent cells (primary hMSCs, p-value = 0.0009; hMSC-telo 1, p-value = 0.0021), whereas a correlation between mean telomere length and percentage of senescent cells was absent (primary hMSCs, p-value = 0.29; hMSC-telo 1, p-value = 0.13). By treating with sub lethal doses and continuing cell growth post exposure, we increased the likelihood that senescence observed in the following population doublings was telomere dependent rather than a result of immediate DNA damage. [179] Telomeres, by virtue of their high guanine triplet content, are more sensitive to oxidative damage and repair less efficiently than genomic DNA. Also, oxidative stress can interfere with telomere maintenance via a suppressive effect on telomerase. [217,218]

Oxidative stress induced telomere shortening occurs in a stochastic manner and can occur to any single telomere in a cell without effecting the mean telomere length considerably, but nonetheless be detrimental to the cell since one critically short telomere alone can trigger cell senescence. Thus oxidative stress can cause immediate (single and double stranded breaks) damage to telomeres resulting in the production of single shortened telomeres. These damaged, excessively shortened telomeres may still be long enough to protect chromosome ends and may therefore not immediately affect cell growth. However, with cell proliferation these telomeres at an earlier time will reach a critically short length, causing premature senescence. Thus, these provide a very early and sensitive marker of oxidative stress induced DNA damage, which is distinct from the gradual decrease in mean telomere length. These critically short telomeres within the cell population were measured by using our novel method U-STELA and were significantly different between

cases and controls indicating the likelihood of a higher level of oxidative stress in subjects who developed cardiac arrhythmia post MI.

Another major finding of this study was the significant increase in telomerase activity in cases when compared to controls and a significant association between telomerase activity and incidence of cardiac arrhythmia. This could represent a protective response to counteract the greater load of short telomeres in the cases, wherein telomerase binds to the shortest telomeres to provide stability. There was no significant association between telomerase activity and the number ICD therapies, excluding the possibility of telomerase increase as a direct consequence of the ICD therapy.

Alternatively, compromised telomeric integrity may play an important role in the context of cardiac arrhythmia in cases and hence the increased telomerase may reflect a telomere independent role as a transcription factor driving expression of key mitochondrial genes and thus protecting the mitochondrial genome from oxidative stress. [217]

<u>All recruited patients did not have a similar profile of telomere biology at birth. The observed difference is due to the effect of genetics and heredity.</u>

Another intriguing possibility is that cases and controls inherited telomeres (and telomerase activity) of different lengths. And hence, the observed difference in telomere biology markers is a result of hereditary differences supplemented by additional attrition secondary to environmental factors. This would also suggest that shorter telomeres are causally related to cardiac arrhythmia in CAD, rather then just being a marker of the disease process.

Genetic determination of telomere length has been suggested in twin and family studies with estimated heritability of approximately 78%. [181,219]

Slagboom et al examined the correlation between monozygotic and dizygotic twins and found a higher correlation in monozygotic twins (0.78) than dizygotic twins (0.39). Given that monozygotic twins are identical, they are intuitively expected to inherit telomeres of the same length regardless of the precise mode of inheritance. Any measured differences in telomere length between the twins at a later date likely reflect differing rates of attrition throughout life. [219]

If there is a primary genetic basis/association determining telomere length, then individuals born with shorter telomeres would be at a greater risk of premature biological ageing, more prone to stochastic and replicative shortening during MI and hence more likely to develop cardiac arrhythmias post MI. And hence telomere biology might just not be a marker of disease process but have a greater bearing in the functional basis and causality of the disease process. The genetic variation in telomere biology markers has been further investigated in chapter 4.

3.4.3 Strengths of the study

The current study is novel in characterising the telomere dynamics of patients at high risk of sudden death. It investigates the association between telomere dynamics in peripheral leukocytes and incidence of cardiac arrhythmia. Moreover, as opposed to studying a single telomere parameter, it simultaneously measures mean telomere length and telomerase activity in all patients (n = 90) and load of short telomeres in a subset (n=30), at the same time point. Not only does this provide an opportunity to potentially examine the clinical utility of this novel marker, but from a basic science perspective, it gives a holistic view of the mechanisms underlying cellular senescence and biological ageing and its role in cardiovascular disease.

The experimental methodology used in this study examines various facets of telomere biology and has several advantages. Telomere length analysis was done using the real time qPCR in contrast to the previously established gold standard telomere restriction fragment (TRF) analysis by southern blot. This had several advantages including (1) greater speed of analysis needing up to 10 hrs to run multiple samples at the same time. Whereas, setting up and running a standard southern blot can take several hours to days. (2) qPCR requires minimal amount of starting material (ng levels) when compared to TRF analysis (µg levels) and hence can be applied to cohorts where DNA may not be readily available, to study specific cell types and facilitates multiple experiments on the same sample amount available. (3) qPCR is specific to true telomeric repeats and hence variation in estimated length due to subtelomeric repeats is significantly less. The coefficient of variation of qPCR can be reduced to less than 4% when using different DNA standard dilutions on each plate. [220] TRF analysis tells little about any particular telomere, and is indicative of the mean telomere length of that particular sample. Different telomeres within the same cell and the same telomere within different cells may have different lengths and this results in the characteristic telomere “smear”. In addition, the smear can also include a variable region of subtelomeric DNA that contributes to the estimate of the mean telomere length. The coefficient of variation can be as high as 5% depending on the restriction enzymes used, which suggests the presence of subtelomeric length polymorphisms and/or subtelomeric restriction site polymorphisms that can confound the TRF length results. [176]

However, the qPCR itself is subject to similar criticism in that it estimates the mean rather than the specific telomere length. Although, the mean telomere

length provides useful information on cellular ageing, the study of specific telomere length is crucial. This is because there is a high degree of inter- and intracellular alongwith inter- and intra chromosomal variation in telomere length as indicated by the wide melt-curves obtained in PCR analysis and the smear seen in TRF analysis. Moreover, the hypothesis that the presence of a single critically short telomere induces senescence, requires a more detailed analysis than just mean telomere length. This was carried out in this study by using the Universal Single Telomere Length Analysis (Universal STELA). This technique enables the measurement of the load and distribution of the shortest telomeres in the entire cell sample. [152,220] Hence, a combination of methods used to assess telomere length enabled quick and accurate analyses of effect of replicative senescence and stochastic shortening on telomere length and hence biological ageing in cardiovascular disease.

The study has clearly established a strong correlation between critically short telomeres and telomerase activity with incidence of VA in ischaemic cardiomyopathy patients. These results are now being used as the basis of prospective, multicentre trials to examine longitudinal changes in telomere biology and their association with cardiac arrhythmia, to confirm or refute the clinical worth of this novel marker in ICD prescription. To the basic scientist, this would provide further support to the building evidence that telomere length is a primary abnormality predisposing to more advanced cardiovascular disease.

3.4.4 Limitations of the study

A number of limitations of this study require consideration. This is a case control study, which gives a cross-sectional analysis of telomere changes at a single time point. Longitudinal changes could not be determined due to the

study design. Hence it is difficult to speculate whether telomere attrition is a primary abnormality predisposing to increased susceptibility to cardiac arrhythmia in this group of patients or if it is just an epiphenomenon. Also, by virtue of this being a cross-sectional study, the effects of certain longitudinal factors might have been deemed non-significant due to large inter-individual variation.

Also, there is a potential for introducing bias due to the study design. The cases included in the study are those who survived a MI and subsequently developed a poor left ventricular function. However, a significant proportion of subjects who have an MI, do not survive the primary event. Not including this group of patients may introduce a survival bias and it becomes challenging to exclude the small chance of longer telomeres conferring a poor survival prognosis post MI.

The ICD is one of the primary data collection tools in this study and also the defining criteria for division between cases and controls. All patients had a primary prevention ICD implant on the background of ischaemic cardiomyopathy and devices were programmed in a similar fashion at implant based on institutional practice. The device programming, to a large extent, determined the incidence of ICD therapy and hence classification of cases and controls in the study. Over a period of follow-up since implant, some patients underwent device programming changes based on their clinical need. This made the group more heterogenous and could have had an impact on study results. However, this was accepted as a potential shortcoming of the retrospective nature of the study design and also the need to prioritise patient safety in a clinical setting.

The mean telomere length was computed by qPCR method. The coefficient of variance for qPCR has been shown to range between 6.4% to 28% in various studies. [220] This is because qPCR is more susceptible to minor variances in sample conditions and DNA integrity. Small variations in sample quality as well as individual donor differences may be amplified by qPCR which could decrease the accuracy of the results. This is a well recognised limitation of qPCR and a smaller sample size in this study could have potentially amplified this problem.

Despite its novelty and advantages, U-STELA method (used for analysing critically short telomeres) is a very laborious method and is not as high throughput as the real time PCR. This limits its utility for large-scale epidemiological studies. Development of a more user-friendly technology is warrantied.

Mean telomere length, critically short telomeres and telomerase activity have been analysed in the whole white blood cell population. A difference in mean telomere length between different subsets of white cells and their variation with gender has been shown in some studies. [221-223] The potential impact of not analysing white cell subsets, can be appreciated by considering the example of atherosclerosis, which was a common factor in both cases and controls in this study. Atherosclerosis is characterised by systemic oxidative stress and low-grade chronic inflammation, mediated by monocytes, macrophages and T-lymphocytes. [224-226] Recruitment of these inflammatory cells varies during the course of the disease. Leukocyte subsets play a crucial role in the age dependent development of atherosclerosis and hence telomere length in leukocytes provides substantial information about cardiovascular ageing in this group of patients. However, this information can vary if whole

white blood cell population is used depending on the stage of the disease process. Moreover, the composition of leukocytes can vary significantly amongst individuals and due to acute infection or comorbidity and even in the same individual from one day to another. Hence telomere length of monocytes, granulocytes or specific T-cell populations may provide more stable results. Better evidence is required to substantiate this.

All telomere analysis has been performed on leukocytes derived from peripheral blood samples. This has not been directly correlated with the primary tissue of interest i.e. cardiomyocyte. Other studies have shown a correlation between leukocyte telomere length and vascular tissue including carotid and aortic endothelium and venous grafts. [155,161] However, the number of these studies are limited and leukocyte telomere biology has not been correlated with other tissues. This correlation is largely presumed on the basis of the limited amount of evidence present and also keeping in mind the primary hypothesis that telomere length is largely inherited and hence individuals with shorter telomeres in peripheral blood samples should also possess shorter telomeres in all other cell types.

Chapter 4 – ASSOCIATION OF GENETIC VARIATION IN TELOMERE RELATED SNP WITH VENTRICULAR ARRHYTHMIAS

4.1. Introduction and Aims

It has already been demonstrated in the previous chapter that leukocyte telomere biology has a significant association with incidence of potentially fatal cardiac arrhythmia in patients with ischaemic cardiomyopathy. Telomere attrition has a significant association with cardiovascular ageing and disease process and is a result of heredity and genetic variation at birth, compounded by environmental factors including oxidative stress and chronic inflammation associated with atherosclerosis. The effect of replicative senescence and stochastic shortening on telomere length was investigated in the previous study (chapter 3).

Telomere length has a strong genetic determination, with heritability estimates ranging from 44% to 80%. [119,227] The telomere hypothesis postulates that shorter telomeres are causally implicated in the pathogenesis of coronary artery disease. Consequently, shorter inherited telomeres could represent a primary abnormality predisposing to an increased risk of cardiovascular ageing. Since a substantial proportion of the marked inter-individual variation in telomere length is genetically determined, [119,219,228] this should be taken into consideration when analysing inter-individual variability. The concept of genetics and heredity of telomere biology has emerged given the high inter-individual variation in telomere length across newborns [229-232] and in individuals of the same age group, telomere length in poorly proliferating cells reflecting the telomere length found during early development, [233]

telomere length comparisons across monozygotic and dizygotic twins and the association of paternal age at conception and telomere length heritability. [233]

A number of genetic loci have been associated with telomere length. As telomerase (reverse transcriptase TERT and ribonucleoprotein TERC) is responsible for maintaining telomere length, factors that influence telomerase activity are clearly important when considering an association between telomere length and clinical phenotype. There is increasing evidence that genetic variation in key genes impact on telomere length and telomerase activity. A growing number of single nucleotide polymorphisms (SNPs) have been associated with telomere length and/or telomerase activity. Some of these include rs12696304, rs16847897, rs10511887, rs610160 (Codd et al); [234] rs2630778, rs11551026, rs2125173, rs10506083, rs10844149 (Mangino et al); [235] rs 2735940 (Matsuabara et al).[236] Other SNPs reported to have an association with mean telomere length in the British Heart Foundation Family Heart Study (BHF – FHS), [237] United Kingdom and Blood Service donors (UKBS) [238] have been shown in Appendix C.

Notably, a genome wide association analysis of mean telomere length (in 2917 individuals with follow up replication in 9492 individuals) identified an association between telomere length on 3q26 (rs 12696304, combined $p = 3.72 \times 10^{-14}$) at a locus that includes TERC, which encodes the telomerase RNA component. Each copy of the minor allele of rs12696304 was associated with a ~ 75 bp reduction in mean telomere length, equivalent to ~ 3.6 years of age related telomere length attrition. This association was thought to be mediated by an effect on TERC expression. However, telomerase levels were not available to substantiate this. [234,237]

Thus genetically determined telomerase expression influences telomere length but its functional impact on somatic cells (where telomerase is largely suppressed) is unknown. Despite several reported associations (SNPs and telomere length), we elected to investigate correlations of SNP12696304 with telomere biology in our study because it has a very strong association with mean telomere length ($p = 3.72 \times 10^{-14}$), is located at a locus including telomerase RNA component TERC and shown to have a significant impact on mean telomere length measurements (each copy of minor allele associated with a ~ 75bp reduction in mean telomere length). [234] Financial constraints were an additional factor limiting the number of SNPs that could be investigated.

Following on from results of the previous study (chapter 3) and the evidence suggesting genetic determination of telomere biology, it was hypothesised that (1) telomerase activity will be different across different genotypes of the SNP 12696304 on chromosome 3q26 (that encodes TERC) (2) differential telomerase expression across genotypes would have an impact on cardiovascular ageing and hence the incidence of VA in this cohort.

To test this hypothesis, the current case-control study was designed to test the association between genetic variation in SNP 12696304 and leukocyte telomere biology (telomerase activity and mean telomere length) with incidence of cardiac arrhythmia in ischaemic cardiomyopathy patients.

4.2. Methods

This case-control observational study was carried out at St Bartholomew's hospital and William Harvey Research Centre. Ethical approval was obtained from the local research ethics committee. All patients gave written informed consent and were recruited from the ICD clinic at St Bartholomew's Hospital.

Investigators were blinded to clinical history and all genotyping was carried out blinded to clinical data.

4.2.1 Subjects

The patients enrolled in this study were the same cohort as described in the previous chapter. The recruitment process, inclusion and exclusion criteria have been described before. SNP genotyping analysis was performed on all the 90 patients recruited to the study (35 cases and 55 controls).

4.2.2 Measurements

Clinical

A standard questionnaire was filled in for all enrolled patients. Clinical details were obtained at recruitment by interviewing the patient and by examining their hospital records. The questionnaire recorded their medical history (to identify hypertension, diabetes, chronic renal failure, hypercholesterolemia, smoking status, family history of ischaemic heart disease and any other significant medical conditions), drug history, NYHA class, details of ICD implant, programming and previous arrhythmia history. These have been detailed in Chapter 2.

At recruitment, the patients also underwent a trans-thoracic echocardiogram to assess left ventricular function (LVEF measured by modified simpsons method), peripheral venous blood sample for general labs (including renal function, inflammatory markers - WCC and CRP, lipid profile), brain natriuretic peptide (BNP) and telomere genotyping.

Genotyping analysis

Genomic DNA was extracted from whole blood as described in chapter 2. Taqman SNP genotyping and allelic discrimination was used to genotype

patients to assess the association of genetic variation in telomere related SNP and ventricular arrhythmias. All samples were analysed blinded to clinical data.

4.2.3 Statistical Analyses

Statistical analyses were carried out using SAS VERSION 9.3 software. Continuous variables are reported as mean ± standard deviation, or median (range) if not normally distributed. Characteristics of cases and controls were compared using chi-squared test for categorical variables and unpaired t-test for continuous variables if normally distributed or Mann-Whitney U test if not normally distributed. The effects of age, gender, WCC, genotype and other individual cardiovascular risk factors on telomerase expression in individual genotypes were assessed using a logistic regression model. The individual genotype (telomere SNP rs 12636904), as a risk factor for appropriate ICD therapy for potential fatal VA (surrogate for SCD), alone and in combination with telomere dynamics telomerase activity with were assessed using logistic regression.

4.3. Results

4.3.1 Demographics

90 patients were enrolled in this study. Of these 35 were in the case arm and 55 in the control arm. This was the same cohort as described in the previous chapter. Their baseline demographic characteristics have been described in chapter 3.

4.3.2 Correlation of ICD Therapy with Genotype

A univariate analysis to examine the association of ICD therapy and the genotype is shown in the table below – table 17.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
SNP C/C	1	-	-
SNP C/G	0.54	0.21 – 1.417	0.343
SNP G/G	0.80	0.198 – 3.236	0.907

Table 17: Univariate correlation analysis of ICD therapy and genotype

No significant correlation between the genotype and ICD therapy was identified (C/C OR 1; C/G OR 0.54, CI 0.21 – 1.417, p-value 0.343; G/G OR 0.80, CI 0.198 – 3.236, p-value 0.907).

An adjusted model again showed no significant correlation between genotype and ICD therapy (C/C OR 1; C/G OR 0.632, CI 0.211 – 1.891, p-value 0.48; G/G OR 0.921, CI 0.194 – 4.374, p-value 0.84) – Table 18.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
SNP C/C	1	-	-
SNP C/G	0.632	0.211 – 1.891	0.48
SNP G/G	0.921	0.194 – 4.374	0.84
Gender	0.845	0.138 – 5.158	0.85
Age	0.938	0.885 – 0.993	0.03

Table 18: Multivariate correlation analysis of ICD therapy and genotype

4.3.3 Correlation of ICD Therapy and Telomere Length in Different Genotypes

There was no significant correlation between mean telomere length and ICD therapy and VA in the present study as shown in chapter 3. The distribution of mean telomere length across different genotypes and its association with ICD therapy in the individual genotypes was investigated.

The mean telomere length was shorter in individuals homozygous for G allele when compared to the heterozygotes and those homozygous for C allele (1.28,1.10,1.18) in cases. These findings are similar to those reported in other studies, although in a different clinical context. [234] The mean telomere length for the entire cohort (cases and controls) was not significantly different between the two homozygous allele groups. This difference only emerged significant in patients who had ICD therapy for potentially fatal VA. However, within individual genotypes, there was no significant difference in telomere length between cases and controls (C/C, C/G, G/G; p-value 0.41, 0.64, 0.71). The association of mean telomere length with ICD therapy in the individual genotypes is shown below.

Correlation of ICD therapy and mean telomere length in genotype C/C

There was no significant correlation between ICD therapy and mean telomere length in the C/C genotype (OR 1.06, CI 0.86 – 1.32, p-value 0.58) as shown in the table below – table19.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Mean telomere length in C/C genotype	1.06	0.86 – 1.32	0.58

Table 19: Correlation of ICD therapy and mean telomere length in C/C genotype

The mean telomere length was not different between cases and controls in genotype C/C (20.58 versus 17.65; p-value 0.41).

Correlation of ICD therapy and mean telomere length in genotype C/G

The mean telomere length did not correlate with the incidence of ICD therapy in the heterozygous cohort (OR 0.91, CI 0.57 – 1.44, p-value 0.68) – table 20.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Mean telomere length in C/G genotype	0.91	0.57 – 1.44	0.68

Table 20: Correlation of ICD therapy and mean telomere length in C/G genotype

The mean telomere length was not different between cases and controls in genotype C/G (15.35 versus 17.02, p-value 0.68).

Correlation of ICD therapy and mean telomere length in genotype G/G

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Mean telomere length in G/G genotype	0.92	0.55 – 1.52	0.73

Table 21: Correlation of ICD therapy and mean telomere length in G/G genotype

Similarly, in individuals homozygous for G-allele, the mean telomere length did not correlate with ICD therapy (OR 0.92, CI 0.55 – 1.52, p-value 0.73). The mean telomere length was not significantly different between cases and controls in genotype G/G (5.4 versus 4.7, p-value 0.71)

The interaction plot for mean telomere length and ICD therapy (cases and controls) across different genotypes is shown in figure 21.

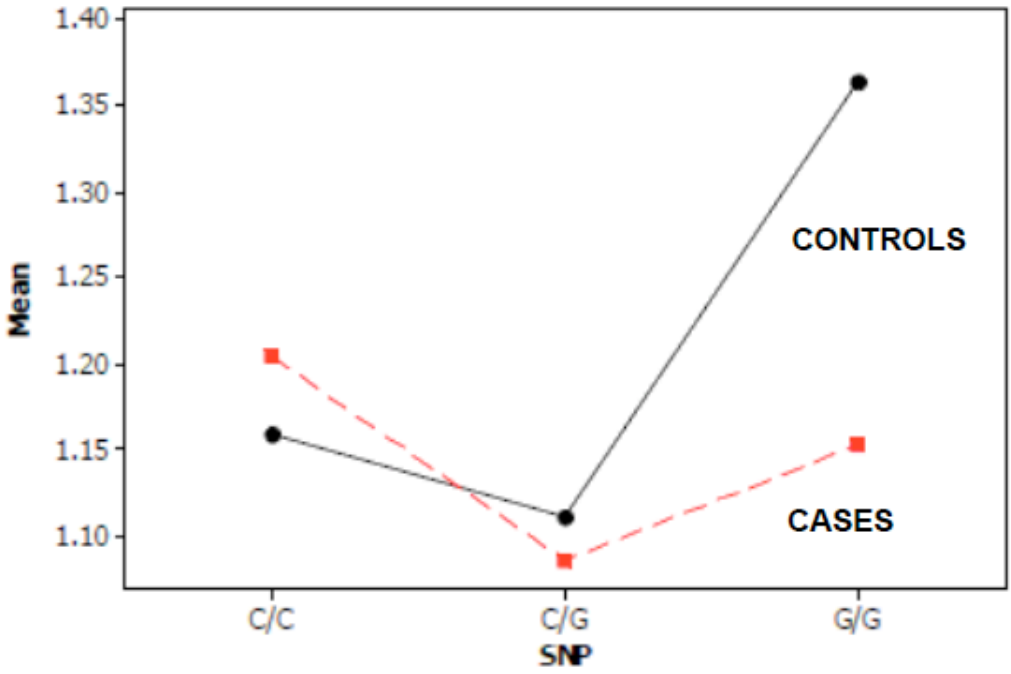

Figure 21: Interaction plot for mean telomere length across different genotypes

4.3.4 Correlation of ICD Therapy and Telomerase Activity in Different Genotypes

A significant correlation between ICD therapy and telomerase activity has already been shown in this study. It was then investigated whether telomerase expression was different amongst genotypes an if there was a correlation between telomerase activity and genotype with VA.

Overall, with cases and controls combined, the telomerase activity was highest in individuals homozygous for C-allele and was similar across the heterozygous allele and individuals homozygous for G allele (C/C 0.35, C/G 0.26, G/G 0.24).

Correlation of ICD therapy and telomerase activity in genotype C/C

The odds of receiving ICD therapy in individuals homozygous for C-allele with increase in telomerase activity were 7.507. There was a strong association between telomerase activity and ICD therapy in the C/C genotype (OR 7.507, CI 1.00-56.61, p-value 0.04) – table 22.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity in C/C genotype	7.507	1.00 – 56.61	0.04

Table 22: Correlation of ICD therapy and telomerase activity in C/C genotype

Correlation of ICD therapy and telomerase activity in genotype C/G

No significant correlation between ICD therapy and telomerase activity was found in the C/G genotype (OR 2.759, CI 0.352 – 21.60, p-value 0.33) – table 23.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity in C/G genotype	2.759	0.352 – 21.60	0.33

Table 23: Correlation of ICD therapy and telomerase activity in C/G genotype

Correlation of ICD therapy and telomerase activity in genotype G/G

The correlation between telomerase activity and ICD therapy was not significant in individuals homozygous for the G-allele (OR 4.137, CI 0.117 – 145.91, p-value 0.43) – table 24.

Parameter	Odds ratio	95% Wald Confidence Limits	p-value
Telomerase activity in G/G genotype	4.137	0.117 – 145.91	0.43

Table 24: Correlation of ICD therapy and telomerase activity in G/G genotype

The interaction plot for telomerase activity with ICD therapy (cases and controls) in different genotypes is shown in figure 22.

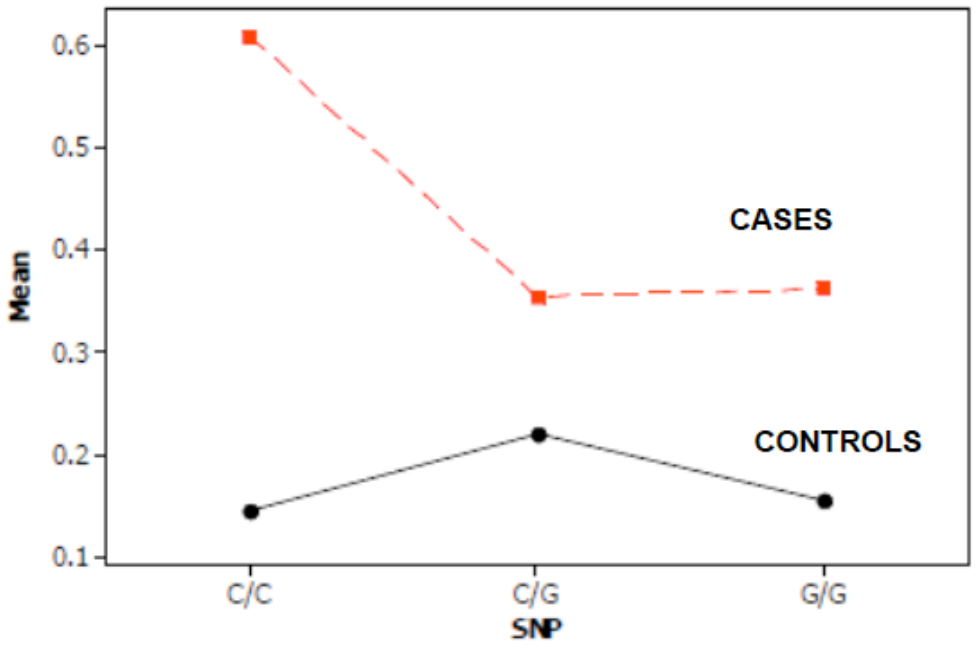

Figure 22: Interaction plot for telomerase across different genotypes

4.4. Discussion

The main finding of this study is that the telomerase expression was significantly higher in individuals homozygous for C allele of a TERC encoding SNP. There is a significant correlation between telomerase activity and cardiac arrhythmia in ischaemic cardiomyopathy patients as shown in the previous chapter. This association remained significant only in the C/C allele of SNP 12696304 whereas individuals who were homozygous for the G allele or heterozygous had similar levels of telomerase across cases and controls. Thus increased telomerase activity could predispose individuals with C/C genotype to a higher incidence of VA post MI. Also, the genotype (independent of the telomerase expression) did not correlate with incidence of cardiac arrhythmia.

Telomere length variability has been attributed to genetic influences. Heritability estimates of 78% have been derived from twin studies and account for inter-individual differences to a large extent. [219] Large-scale genotyping studies have identified various genetic variants which affect telomere length as they harbor genes that encode proteins with known function in telomere biology. These include TERC locus on 3q26, [234] TERT on 5p15.33, NAF1 (nuclear assembly factor 1) on 4q32.2, OBFC1 (oligonucleotide/oligosaccharide-binding fold containing 1) [239] and RTEL 1 (regulator of telomere elongation helicase 1) on 20q13.3. [240-243] TERC and TERT are components of the telomerase ribonucleoprotein moiety, whereas NAF1 is required for H/ACA box snoRNA assembly, the RNA family to which TERC belongs. [240] Therefore, the three most significantly-associated loci (3q26, 5p15.33 and 4q32.2) harbor genes involved in the formation and activity of telomerase. And hence, the genetic basis for this variation in telomere length

is largely due to the presence of telomerase gene variants with variable activity in germ-line and/or stem cell pools.

Of all the genetic loci associated with telomere length we studied the one with the most significant association – rs12696304, which is located 1.5kb downstream of TERC. It has been previously shown that each minor (G) allele of rs1296304 is associated with a shorter mean telomere length equivalent to ~ 3.6 years (75bp reduction) of average age-related telomere attrition. [234] Our study showed similar results with the mean telomere length being shorter in individuals homozygous for the minor allele G. However, this was only true for the individuals who experienced ICD therapy for potentially fatal VA (cases) and not for the entire cohort. Also the magnitude of difference was much smaller, likely due to a much smaller sample size.

The most significant associations of various gene loci with telomere length encode TERC or TERT and their functional basis is believed to be mediated through telomerase expression. However, significant associations between telomerase activity and SNP variants have not been shown in previous studies and all inferences have been derived from quantitative assessment of mean telomere length. The present study shows that the telomerase activity was significantly higher in individuals homozygous for C-allele (compared to homozygous G or heterozygous status) of SNP 12696304, which encodes TERC. Also, there was a significant correlation between risk of VA and increase in telomerase activity in the C/C genotype. This correlation was not significant in the C/G or G/G genotype. Hence homozygosity for C-allele significantly effects telomerase expression in ischaemic cardiomyopathy patients.

As telomerase is responsible for maintaining telomere length, factors that influence telomerase activity are clearly important when considering the association between telomere length and clinical phenotype. There is increasing evidence that genetic variation in key genes has an impact on telomere length, which is largely due to the effects exerted by the enzyme telomerase. The present study shows (1) a significant correlation between a telomere related SNP 12696304 and telomerase activity and (2) a significant correlation between telomerase activity and VA in individuals homozygous for the major allele (C) of the same SNP 12696304 post myocardial infarction. Thus, here is a genetic variant that effects telomerase expression (and hence telomere length) and is significantly associated with the incidence of cardiac arrhythmia in individuals with ischaemic cardiomyopathy.

Given the above findings, it would now be intuitive to try and establish if this association is causal in nature. There is no doubt that homozygosity for C allele significantly effects telomerase expression, which in turn is significantly associated with ICD therapy (VA). However, the important question is: Does a high telomerase activity predispose C homozygous individuals to develop VA or is the increase in telomerase activity in C homozygous individuals secondary to development of VA? When answered, not only will this establish the role of telomere biology and its genetic variation as a primary mechanism in causality of cardiovascular ageing; but also help develop these markers as potential predictors of cardiac arrhythmia in ischaemic cardiomyopathy.

It is difficult to answer this question conclusively within the realms of the current study. This is largely due to the absence of data depicting longitudinal changes in telomere biology in this group of patients, given the study design. However, we can hypothesise the cause behind this association and its

potential implications can be speculated with reasonable accuracy. Potential hypotheses include:

Increase in telomerase is a result of ICD therapy

The rise in telomerase activity can be the result of ICD therapy itself. Patients received ICD therapy for potentially fatal VA. Both the cardiac arrhythmia or ICD therapy itself could potentially have an impact on cellular function and stability leading to an enzyme rise, which is independent of telomeric function.

However, if this were the case, then one would expect the telomerase levels to be proportionate to the number of ICD therapies received. There was no significant correlation between telomerase activity and number of ICD therapies in this cohort. Also, if the telomerase rise was solely the result of ICD therapy, then it would be difficult to explain why this association was seen predominantly in one genotype (homozygous C) despite it not including the patient with the maximum number of shocks. Nonetheless, conclusions can only be drawn with the availability of longitudinal changes in telomerase, before and after ICD therapy, from large-scale prospective studies.

Increase in telomerase is a protective response to critically short telomeres

Myocardial infarction, a major complication of atherosclerosis/CAD, is associated with very high degrees of oxidative stress. As per our primary hypothesis (Chapter 1), telomere shortening at the time of MI, results in poor myocardial repair process and predisposes patients to greater arrhythmic tendency. This is mediated by the formation of critically short telomeres (shown to be higher in cases in this study) as a result of stochastic shortening. A single critically short telomere can lead to cellular senescence by virtue of genomic instability.

The observed increase in telomerase may be a protective response to stochastic telomeric shortening to maintain genomic stability and prevent cellular senescence. This is further supported by the fact that both telomerase activity and load of short telomeres followed a similar trend – higher in cases versus controls and highest in individuals homozygous for C-allele of SNP 12696304. However, it is difficult to be certain at what time point after MI do telomeres become critically short and precipitate a rise in telomerase activity. Whether this happens before or after the development of left ventricular dysfunction, could have important implications in clinical risk stratification of SCD in ischaemic cardiomyopathy.

Genetic variation predisposes some individuals to express telomerase at the time of index event in anticipation of later instability (critically short telomeres)

A very interesting possibility would be individuals being born with a certain predilection (mediated by telomere biology) to develop cardiac arrhythmias, should they suffer from a MI. This would account for genetic variation in telomere dynamics in these individuals. So, the increase in telomerase activity in C homozygous alleles of the SNP encoding TERC would be present prior to the development of VA. The increased telomerase expression in these individuals might be secondary to an inherited variation in TERC, which only becomes functional (increased telomerase activity) on exposure to extreme oxidative stress (MI). And it is this genetic variation that predisposes them to a greater arrhythmic tendency post MI.

Poor left ventricular function and MI are categorical traits, which might change with age or during the course of a disease process (CAD). However, genetic variation in TERC/Telomerase expression would be present at the time of genotyping - before (or after) the development of left ventricular dysfunction

or cardiac arrhythmias. These can thus be accurately quantified early on in the disease process. Thus, if increased telomerase expression is present in C homozygous alleles of the studied SNP, before development of left ventricular dysfunction post MI, then this can be used as an intermediate phenotype to identify individuals prone to VA relatively early on post MI. Again, this needs prospective studies to confirm the observed associations and develop telomere biology markers for clinical use.

This study provides novel insights into genetic determination of telomere biology and its association with cardiac arrhythmia in ischaemic cardiomyopathy patients. The main findings suggest that variants in genes encoding proteins with known function in telomere biology have a functional effect and can modify clinical phenotype. Telomere biology markers can be developed as an intermediate phenotype, as a quantitative marker, to assess risk of cardiac arrhythmia post myocardial infarction, early on in the disease process.

Chapter 5 – GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 Overview of the Project

The primary aim of this study was to characterise telomere biology in patients with ischaemic cardiomyopathy and at a high risk of sudden death. This was to investigate associations between telomere biology and cardiac arrhythmia and to explore their potential for translation into clinical use for risk stratification of sudden death. The main findings of the study indicate that load of short telomeres is greater and telomerase activity higher in individuals who experience potentially fatal VA than in those who do not. Also, these telomere markers have a significant correlation with incidence of VA. Thus proving the primary hypothesis of the study, stipulating an association between telomere biology and VA occurrence post myocardial infarction. Also, genetic variation in telomerase expression was found on genotyping a TERC encoding SNP and this differential expression across genotypes correlated significantly with incidence of VA in the major homozygous allele.

Two novel methods were used to assess telomere attrition. The widely used real time PCR method assesses the mean telomere length. Although this is a very quick method with a high throughput and requires minimal amount of starting genetic material, it does have its shortcomings. This is a somewhat crude analysis of telomere length, and doesn't give much information on cell or chromosome specific telomere length. The analysis of specific telomere length is important because: (1) there is a high degree of inter- and intra-chromosomal, in addition to inter- and intra-cellular differences in telomere length as depicted in the wide melt curves obtained during real time PCR analysis. (2) increasing evidence suggests that cellular senescence is not

solely a result of gradual decline in telomere length. A single critically short telomere (4-7kb) can induce senescence. [244] Moreover, chromosome specific patterns of telomere length have been shown, [245] suggesting that there might be a chromosome specific telomere length profile that is associated with cardiac arrhythmia in ischaemic cardiomyopathy. Or there might be a specific telomere that is consistently short in our patient group – similar to the short telomere on chromosome 17p (harbouring p53 and tumour suppressor genes) that is lost in human cancers. (3) Telomere shortening can result in altered gene expression as a result of telomere position effect and hence necessitating the study of specific telomeres. Our novel method U-STELA offered a solution to the shortcoming of the gold standard real time PCR. It enabled to determine the difference in telomere length between cases and controls by analysing the critically short telomeres regardless of their chromosomal location. Other methods of telomere analysis are unable to detect these short telomeres as they use mean telomere length or telomere lengths of dividing cells and therefore non-senescent cells. With U-STELA it was possible to quantify the critically short telomeres and directly investigate their relationship with cellular senescence in this clinical phenotype. The method of quantification of the short telomeres is novel in that the number of short bands below a certain threshold were counted and expressed as a number relative to DNA concentration of each cell. Such a threshold parameter serves as a surrogate measure for the frequency within individual cells of telomeres that are no longer able to protect the chromosome ends.

Telomere length at any given point in time is a function of initial length and the rate of attrition. Different mechanisms of telomere shortening and factors effecting telomere length were investigated in this study. Genetic

variation in a SNP lying 1.5kb downstream of TERC was shown to have an impact on telomerase activity. Telomere shortening as a result of replicative senescence was reflected in the mean telomere length whereas stochastic telomere shortening was determined by quantifying the load of short telomeres. These mechanisms of telomere attrition in this clinical cohort represented the cumulative inflammatory and oxidative burden at the cellular level.

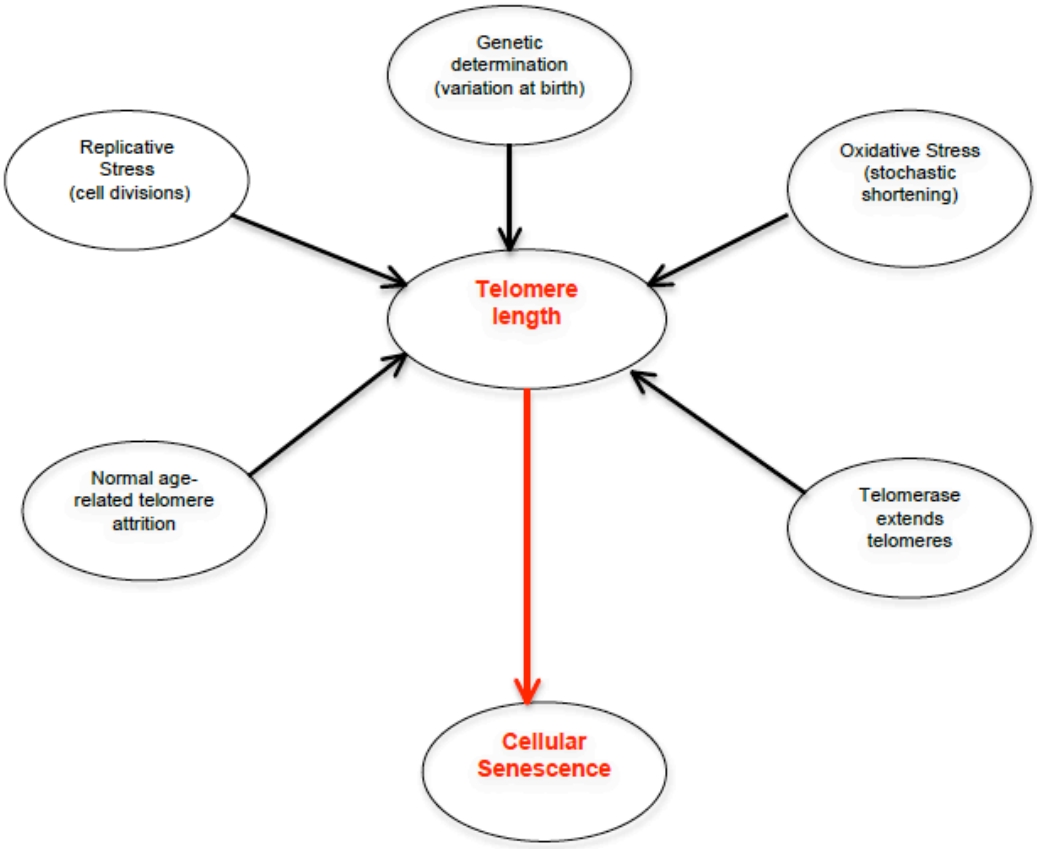

Figure 23: Mechanisms of telomere shortening

Genetic variation in telomerase and its association with cardiac arrhythmia has been well demonstrated in the present study. Whether the observed difference in telomerase expression was present at birth or the genetic variant was only manifested as a functional change after myocardial infarction, is difficult to established within the realms of this study. Previous

studies have shown that telomerase might account, in part, for the high estimate of heritability. A burst in telomerase activity during embryogenesis (morula t blastocyst stage) leading to resetting of telomere length to a defined set point has been shown. [246] Similarly, it has been shown that the telomere length is the same across different foetal tissues at birth. This synchrony amongst foetal tissues implies that from blastocyst stage onwards all cells would turnover at the same rate until birth. [247] If the telomerase activity is the same in all individuals, the telomere length after the “burst” will be a function of length before it. However, if two individuals possess variants of telomerase gene (with different rates of functional activity) then the length set during this “burst” may differ.

In this study an association between systemic telomere biology and clinical disease severity has been established. Critically-short telomeres are the prevailing products of stochastic shortening (likely due to oxidative stress) and whether the increased load-of-short telomeres in patients suffering VA are the result of ICD shock itself or on-going ischaemic insult, which then increases susceptibility to VA is not clear. Nevertheless these findings have major implications for studies examining the pathways from exposure of cells to oxidative stress to the induction of cellular senescence, because a telomeric role can potentially be missed if a method insensitive to changes in single telomeres is applied. Mean telomere length is a crude measure of telomere dynamics and the load-of-short telomeres may be a superior discriminatory marker in patients with ischaemic cardiomyopathy and poor LV function. It is also important to consider, the very interesting possibility, of genetic variation in telomerase expression and its functional impact on clinical phenotype as shown in this study.

Cardiac arrhythmia post MI is a categorical trait and can change during the lifetime of an individual. But can a quantitative trait like telomere length (mean telomere length or load of short telomeres) and telomerase activity be measured with relative accuracy to predict cardiovascular ageing? Could we use telomere biology as an intermediate phenotype to identify individuals at a high risk of cardiac arrhythmias early on in the disease process? Could further research develop telomere biology as a potential biomarker for cardiac arrhythmias?

5.2 Telomere Dynamics as Potential Biomarkers

Leukocyte telomere biology is generally considered as a marker of cellular senescence and biological ageing. It has been shown to have a significant association with cardiovascular ageing especially in the context of atherosclerosis and myocardial infarction. The present study establishes a significant association between telomere dynamics (critically short telomeres and telomerase activity) and incidence of potentially fatal cardiac arrhythmias (sudden death) in ischaemic cardiomyopathy. Intuitively, if this association is validated in a longitudinal study, telomeres could potentially be developed as biomarkers for risk stratification of sudden death in this group of patients. Telomere biology changes progressively with chronological age, has inherent variation amongst individuals, quantifies accelerated biological ageing resulting from replicative senescence and oxidative stress. So does telomere biology have the potential of a robust biomarker of cardiovascular ageing?

In an attempt to answer this pivotal question, the current evidence was examined against a set of standardised well-defined biomarker of ageing criteria developed by the American Federation of Ageing Research: [248,249]

Criterion 1: *It must predict the rate of ageing. In other words, it would tell exactly where a person is in their total life span. It must be a better predictor of lifespan than chronological age.*

To fulfill this criterion, telomere attrition should shorten an individual's life span and should be a better predictor of the same than chronological age. Several studies have reported shortened telomeres and an increased risk of cardiovascular mortality. [180,250,251] This has also been shown in prospective twin studies wherein it was found that the twin with the shorter telomeres had a threefold higher mortality risk than the co-twin over an average follow up period of seven years, regardless of zygosity. [252]

These mortality studies provide good evidence to develop telomere biology as a marker of cellular senescence and cardiovascular ageing. Survival bias in older cohorts might be a potential confounding factor. Whether telomere attrition predicts mortality awaits results of further research from longitudinal studies.

Criterion 2: *It must monitor a basic process that underlies the aging process, not the effects of disease.*

Increasing evidence suggests that telomere biology correlates with biological ageing and the pathogenesis and prognosis of cardiovascular diseases. Heart disease is believed to represent a disease of premature biological ageing and telomere length, independent of chronological ageing, is predictive of an increased of cardiovascular risk. The accelerated biological ageing results from a combination of replicative senescence and a more stochastic telomere loss mainly due to oxidative stress.

In the present study we demonstrated a significant association between an increased load of critically short telomeres and increased telomerase activity with a higher incidence of potentially fatal VA in patients post myocardial infarction with poor left ventricular function. Previous studies have shown a significant association between telomere biology and CAD, [95-103] premature myocardial infarction, [96] decreased renal function in patients with heart failure, [157,158] poor left ventricular function in the elderly and various cardiovascular risk factors including hypertension, [163] diabetes, smoking and obesity. [162]

The cause-effect relationship of cardiovascular diseases and telomere attrition is not very well established. However, there are studies that have distinctly shown that short telomeres distinctly precede the development of clinical disease and hence short telomeres are not just an epiphenomenon. [98] Despite the strong correlation between telomere biology and cardiac arrhythmias in our study, we could not prove the causality due the lack of longitudinal data. This is true for the vast majority of telomere studies as they are cross-sectional in design.

The evidence presented above favours telomere biology as a promising biomarker of cardiovascular ageing. However, this evidence is not absolute and needs further validation in large-scale prospective studies. Given the wide inter-individual variation in telomere dynamics, rates of change will be more informative and relevant to clinical practice than single time-point absolute estimates.

Criterion 3: *It must be able to be tested repeatedly without harming the person. For example, a blood test or an imaging test.*

Currently, the vast majority of telomere analysis is done on peripheral blood samples. This is easy to obtain and has no potential harm to the patient. It is

generally believed that telomere length measured from peripheral blood correlates strongly and is a surrogate for telomere analysis in the tissue of interest. This belief generally stems from the primary hypothesis that telomere length is largely inherited and hence individuals with shorter telomeres in peripheral blood should also have shorter telomeres in other cell types. There is some evidence that this is true and leukocyte telomere length has been shown to be associated with tissue specific telomere length. [253] However, other studies suggest that this might not be the case for all tissue types, especially those with limited cellular turnover. [254]

Given the current evidence, telomere biology currently fulfills this criterion of a biological marker. However, it is likely that this will be challenged as more studies are carried out using different tissue samples.

Criterion 4: *It must be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans.*

The role of telomeres in ageing has been investigated in animal models, mainly mice. Telomere and telomerase has been shown to have a role in the pathophysiology of ageing and ageing related diseases in mice. Telomeres shorten in the mouse with increasing chronological age. [255,256] Moreover, telomeres of telomerase knockout mice show progressive shortening over generations, with later generations demonstrating evidence of cardiac dysfunction, [257] hypertension, [258] premature ageing and a shortened life span. [259] Overexpression of telomerase in mice may contribute to extended lifespan. [260]

However, the available evidence is limited and fundamental differences in telomere biology between mice and humans cannot be ignored. Inbred mice strains have extremely long telomeres when compared to humans (20-150kb vs 15kb); [261] telomerase is easily detected in somatic cells in mice, whereas it is mostly active in germ cells in humans and certain species of mice are known to have a very short life span despite very long telomeres and hence suggesting a different mechanism of ageing. [262,263]

Sufficient evidence is lacking to accept or refute the mouse model for investigating telomere biology prior to validation in humans. Further animal studies on the mouse and use of additional animal models are required to get a clearer picture.

Overall, it is apparent that the evidence supporting the hypothesis that telomere biology is a biomarker for cardiovascular ageing is equivocal. Further evidence is required from longitudinal study designs, which can provide very useful information on intra-individual telomere biology over a life span. These would be far more useful than cross-sectional analyses, which are a reflection of inter-individual variation. In addition to the above considerations, various factors that effect telomere biology comparability across individuals have to be taken into account: [220]

Telomere length inheritance and telomere hypothesis: in the present group of ischaemic cardiomyopathy patients, two main hypotheses govern telomere attrition by virtue of their correlation with coronary artery disease. The first hypothesis eludes to accelerated leukocyte telomere shortening secondary to stochastic shortening (likely due to oxidative stress) and increased replicative senescence (due to increased inflammation) post myocardial infarction and/or in stable coronary artery disease. [224,264] The alternative hypothesis, commonly

known as “telomere hypothesis” postulates that shorter telomeres may be causally implicated in the pathogenesis of coronary artery disease. [98] Consequently, shorter inherited telomeres could represent a primary abnormality predisposing to an increased risk of cardiovascular ageing. And hence, when interpreting inter-individual telomere variability, it might be important to consider the initial telomere length at birth.

Genetic variation in telomerase expression: telomerase dependent telomere variability has been shown in large-scale genome wide association studies. A significant association of leukocyte telomere length with single nucleotide polymorphisms at a locus that includes telomerase RNA component TERC. [219,234,265] Hence, not only are there inherited differences in telomere length, but genetically determined telomerase activity has an impact on telomere length variability. This is then further compounded by the effect of environmental factors like inflammation and oxidative stress.

Population based comparability: A substantial difference in telomere lengths across different populations and ethnicities has been shown. [220] In fact, ethnicity is a significant confounding factor in telomere studies. The European Atherosclerosis Research Study II, showed a significant variation in telomere lengths across eleven European countries. The telomere length varied between 5.1 kb to 18.6 kb. [266] This vast variation across populations and different ethnicities poses a challenge for telomere comparisons across populations and generalisability for its use as a biomarker.

Longitudinal measurements of telomere biology: the growing evidence that signifies the association of telomere biology with cardiovascular ageing (biological ageing) is derived from cross-sectional studies. These allude to telomere measurements at a single time point. However, given the dynamic

nature of coronary artery disease and its varied time course, longitudinal changes in telomere dynamics associated with disease process would intuitively be more useful.

A few studies have investigated changes in telomere dynamics in coronary artery disease prospectively. The Heart and Soul study measured leukocyte telomere length at baseline and after 5 years in patients with coronary artery disease. As expected, telomere attrition was noted over a time period of 5 years (5.5 kb at baseline to 5.3 kb at 5 years) with an average annual loss of 40 bp. However, it was also noted that telomere shortening was only evident in 50% of the patients. The remaining had either no change in their telomere length or demonstrated an increase in the same. [267] Another study measured leukocyte telomere length over three different time points over 12 years and concluded that the average annual rate of telomere attrition is 31 bp and telomere length elongation is a methodological artefact rather than a real phenomenon. [268]

Individual telomere length fluctuations might be a natural component of age related telomere attrition. These changes in telomere biology over time and their association with the disease process need to be established to allow comparison across different cohorts and to translate it into clinically useful information. These would of course, be further influenced by other factors including methodology of telomere measurement, composition of leukocytes at any point in time and the short half life of myeloid cells.

Composition of leukocytes: The vast majority of telomere studies have been done on whole blood cell leukocyte. However, the composition of leukocytes has been shown to vary during the course of atherosclerosis with varied

number of monocytes, granulocytes and lymphocytes being recruited during the course of this inflammatory process.

Role of infection: recent evidence suggests accelerated CD8-Tcell telomere shortening in CMV seropositive patients with coronary artery disease. CMV infection induces a strong reduction of telomere length in the circulating T-cell pool, which is largely attributed to the changes in composition of T-cells. [269,270] CMV seropositivity is evident in 70% of the adult population, making this observation even more significant for analysis and comparability of telomere length.

Methodological errors in measuring telomere biology: leukocyte telomere length has been measured by a number of different techniques including TRF southern blot analysis, qPCR, Flow-FISH etc. The co-efficient of variation for telomere length measurements by different methods varies between 2 to 28%. Even a small measurement error resulting in a co-efficient of variation of 2.5% can result in significant misclassification of individual telomere length. [268] The advantages and disadvantages of various techniques need to be considered to allow uniformity in telomere measurements and to ensure reliability in telomere comparisons across different cohorts.

There is enough evidence that telomere biology can be established as a biomarker for cardiovascular ageing. To achieve this, the following conditions for this marker should be met: [220]

- Telomere biology should be measured in one or more leukocyte subsets to avoid short-term changes in leukocyte composition and its impact on longitudinal variability.

- The average annual telomere loss in the studied cell subset should be greater than in other cell types to reduce methodological measurement errors in telomere length analysis. For instance, populations which lose 40bp/yr are more likely to distinguish an ageing effect than those that shorten at 20bp/yr.
- Longitudinal changes in telomere biology should be characterised in prospective studies with the aim to confirm its association with cardiovascular ageing. This should be done across different population and ethnic cohorts.
- In addition to chronological and biological ageing, effects of oxidative stressors and systemic inflammation on stochastic telomere shortening need to be investigated further. These processes are hallmarks of cardiovascular ageing and their effect on telomere biology needs to be established.
- An internal standard (control) should be used to account for genetic and population based variability. This reference cell population should age slower than the marker population.
- An age limit might exist where telomere length can distinguish between healthy and “prematurely” aged cells, given that telomeres have reached a critically short length or the experimental method used can not separate between two very short telomeres. This possibility needs to be considered and appropriate methods used simultaneously.

5.3 Future Directions

Telomere biology in cardiac arrhythmia and cardiovascular ageing requires further understanding if it is to enter widespread clinical practice. The findings of the present study provide several new avenues for future research.

Having shown a significant association between systemic telomere biology and cardiovascular disease severity, it is vital to determine if this relationship is causal or not. This could be done by characterising longitudinal changes in telomere attrition and telomerase activity in large-scale, multicenter, prospective studies and correlating them to the development of cardiac arrhythmia in ischaemic cardiomyopathy. Whether telomere attrition/telomerase activity are simply biomarkers or whether the observed association has a functional basis remains to be determined. This requires further study of the interaction between telomere biology and functional cardiac electrophysiology and cardiac remodeling after MI. Possible explanations for the underlying mechanism include telomerase mediated prolonged lifespan of inflammatory cells promoting ischaemia or prolonged lifespan of peri-infarct cardiac cells promoting anisotropy and arrhythmic potential. Comprehensive mechanistic studies are needed to investigate this further.

All telomere analysis in this study has been done on genomic DNA derived from peripheral blood leukocytes. This is also true for the vast majority of telomere studies published so far. It is well known that atherosclerosis is characterised by low-grade chronic inflammation and systemic oxidative stress. These are mediated by different leukocyte subsets and recruitment of these inflammatory cells varies during the course of the disease. Leukocyte populations have a crucial role in age dependent development of atherosclerosis, wherein the disease process is mediated by monocytes,

macrophages and T lymphocytes. [271] Telomere shortening is believed to occur at a faster rate in lymphocytes (than in granulocytes) [221,231] and the persistent inflammatory state in atherosclerosis can lead to increased proliferation of lymphocytes leading to a shift towards a more mature populations and hence shorter telomere lengths. [221] This is also seen with the normal ageing process. Concentrations of monocytes and granulocytes in peripheral blood are predominantly elevated in acute MI, but could be high in stable CAD as well. [272] Granulocytes have a relatively short lifespan, lack proliferation and are not known to express telomerase (with the exception of coronary plaque neutrophils). [273] Given the characteristics of our clinical cohort, time lapsed post MI and the associations observed, lymphocyte subset might be a more relevant marker of telomere biology. Total leukocyte telomere length might be unattractive given that leukocyte composition changes in individuals with age and also over the time course of the underlying disease pathophysiology in this cohort.

Telomere biology shows considerable variation across different ethnicities. Race and ethnicity have shown to be significant confounding factors while assessing telomere length. Also, there is considerable genetic variation and heritability involved in telomere assessment. If this were to be established as a biomarker, it would be essential to standardise this for individual ethnicities or across groups of ethnicities. Moreover, the genetic variation in telomerase expression shown in this study is likely to be effected significantly by the race and ethnicity of the population being studied. This needs further thought.

Another important question that requires investigation is the correlation between telomere analysis in peripheral blood and cardiomyocytes. Although

leukocytes provide an easily accessible source for genomic DNA analysis, the relevance of measurement of telomere biology in peripheral blood to various processes that occur in cardiomyocytes at the time of an infarct have not been established so far. There is limited data that suggests that a correlation exists between telomere lengths in different tissues of an individual that indicate the genetic determination of telomere length**.** [274] Some studies have shown the correlation of leucocyte telomere biology in circulating cells and vascular tissues. However, the number of these studies is small and evidence inconclusive. This is an exciting area for further research.

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APPENDIX

A. U-STELA GELS

Load of short telomere analysis on 30 patients is shown below. Figures include annotated gel images and corresponding raw data. Telomere count was done using Vision Works LS Acquisition and Analysis Software (Ultra-Violet Products Ltd, AH Diagnostics). The number of PCR products with a length less than 1500 base pairs (bp) were counted and calibrated based on the PCR template concentration. The load of short telomeres was presented as the number of telomeres below a length of 1500 bp per genome equivalent of template DNA.

GEL 1

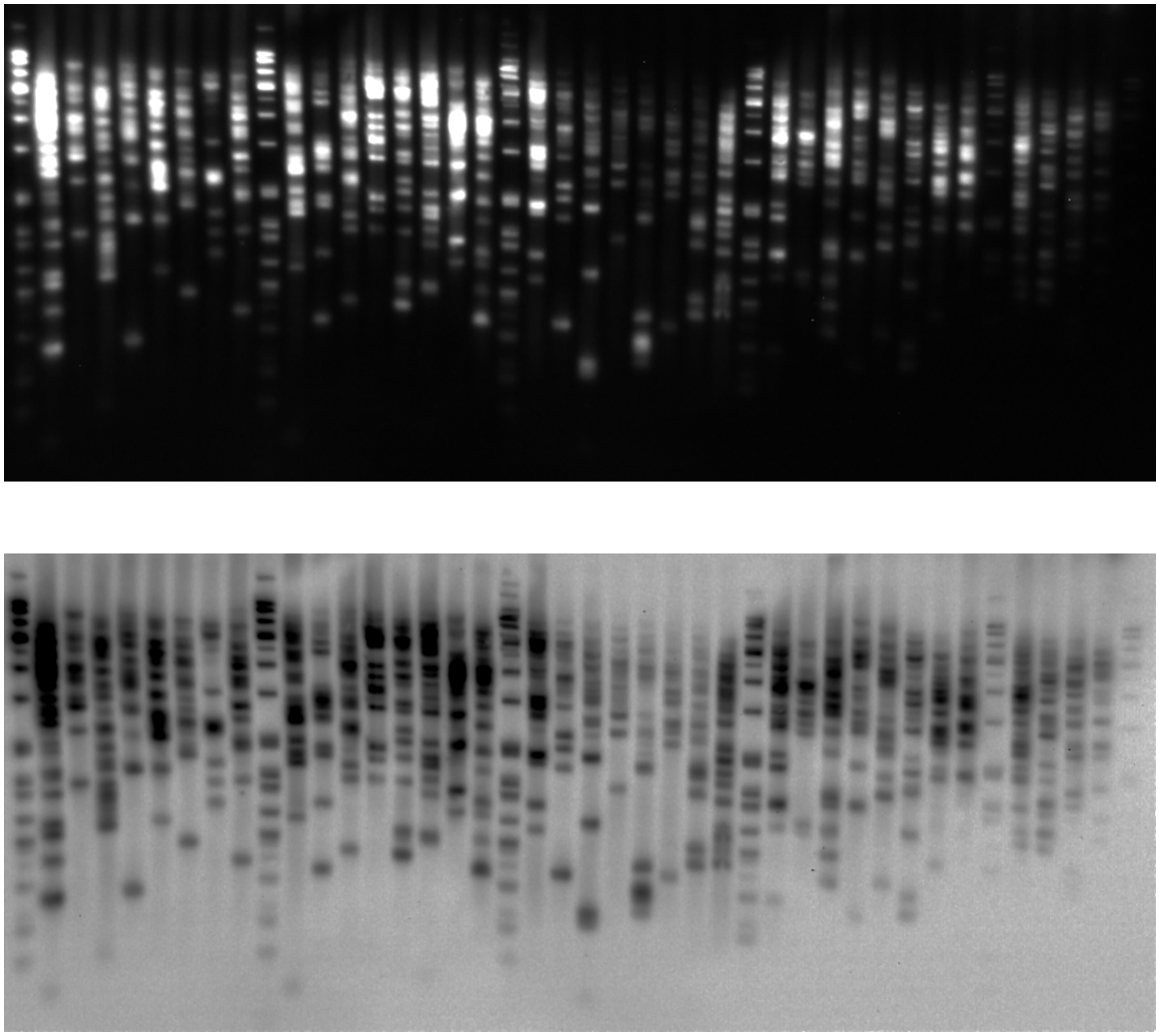

Figure 24: U-STELA Gel 1

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 1_1

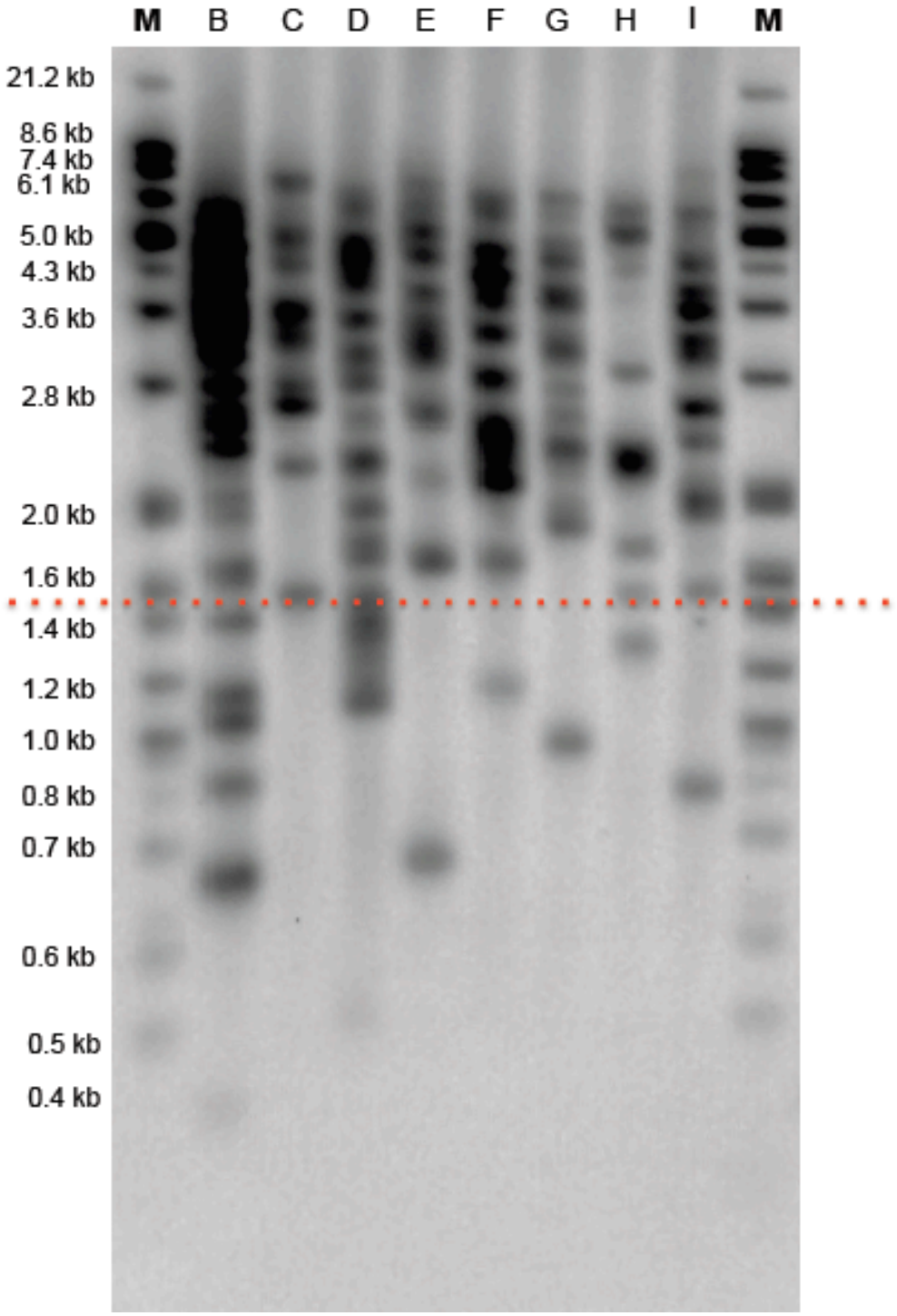

Figure 24(a): U-STELA Gel 1_1 with markers

GEL 1_2

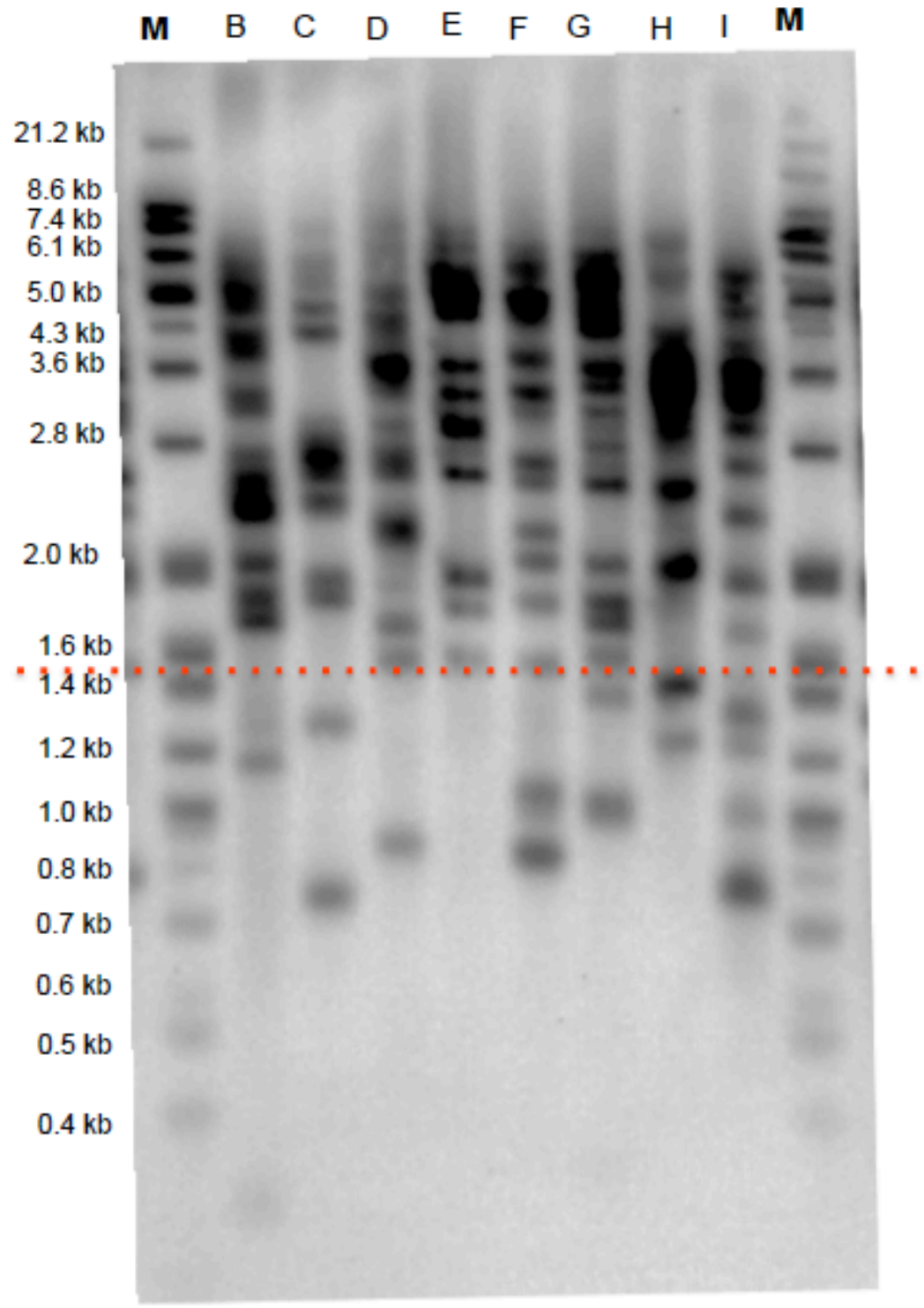

Figure 24(b): U-STELA Gel 1_2 with markers

GEL 1_3

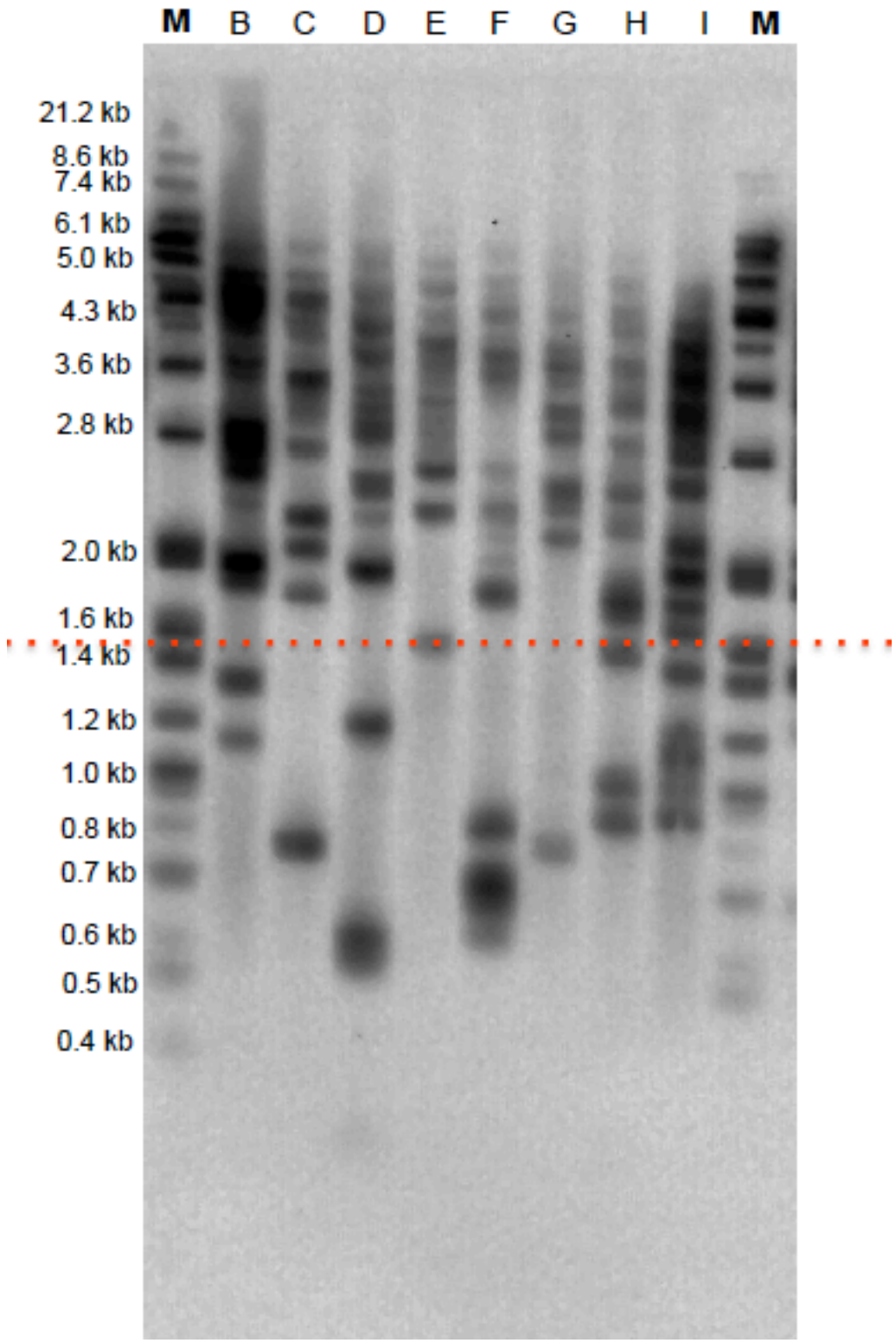

Figure 24(c): U-STELA Gel 1_3 with markers

GEL 1_4

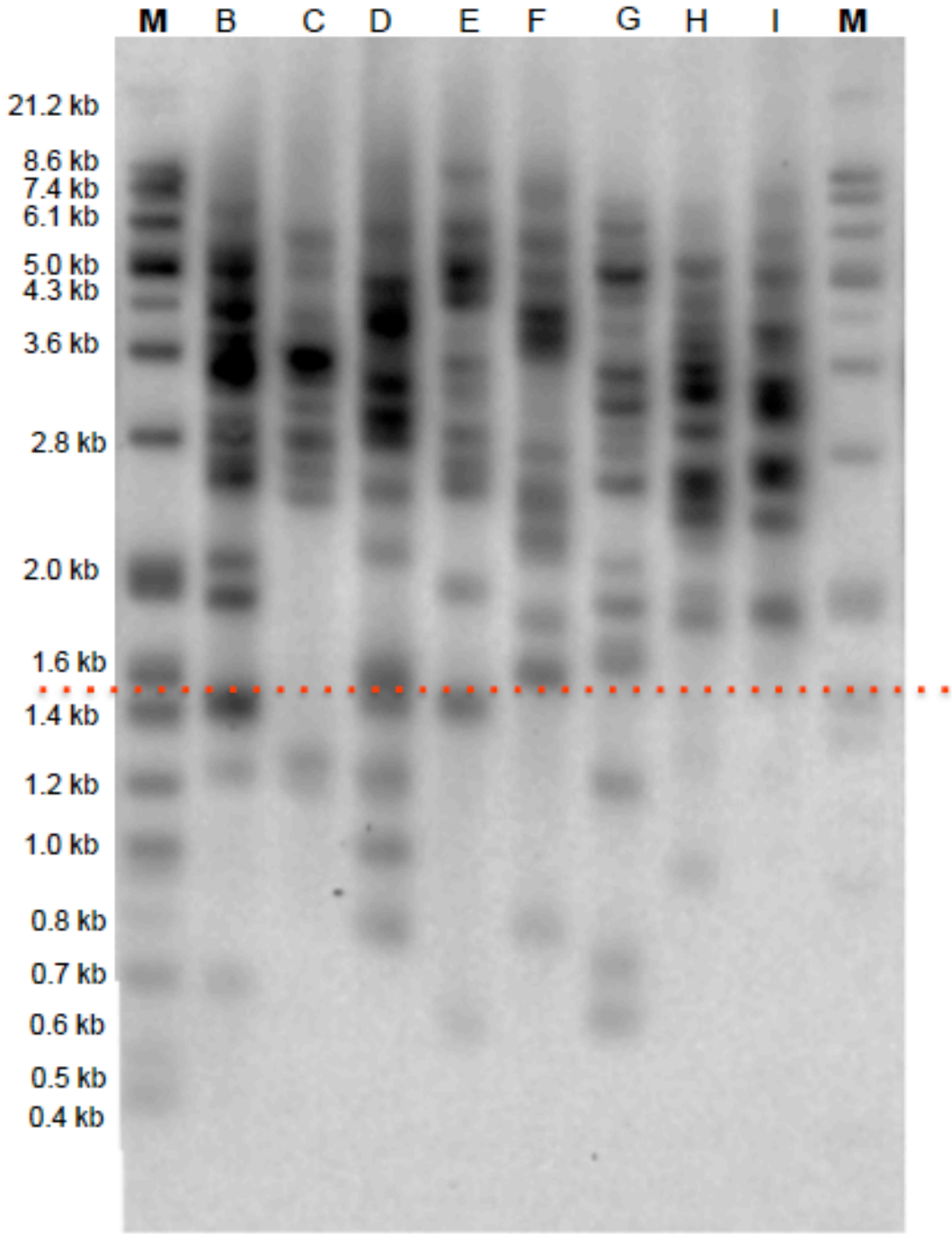

Figure 24(d): U-STELA Gel 1_4 with markers

GEL 1_5

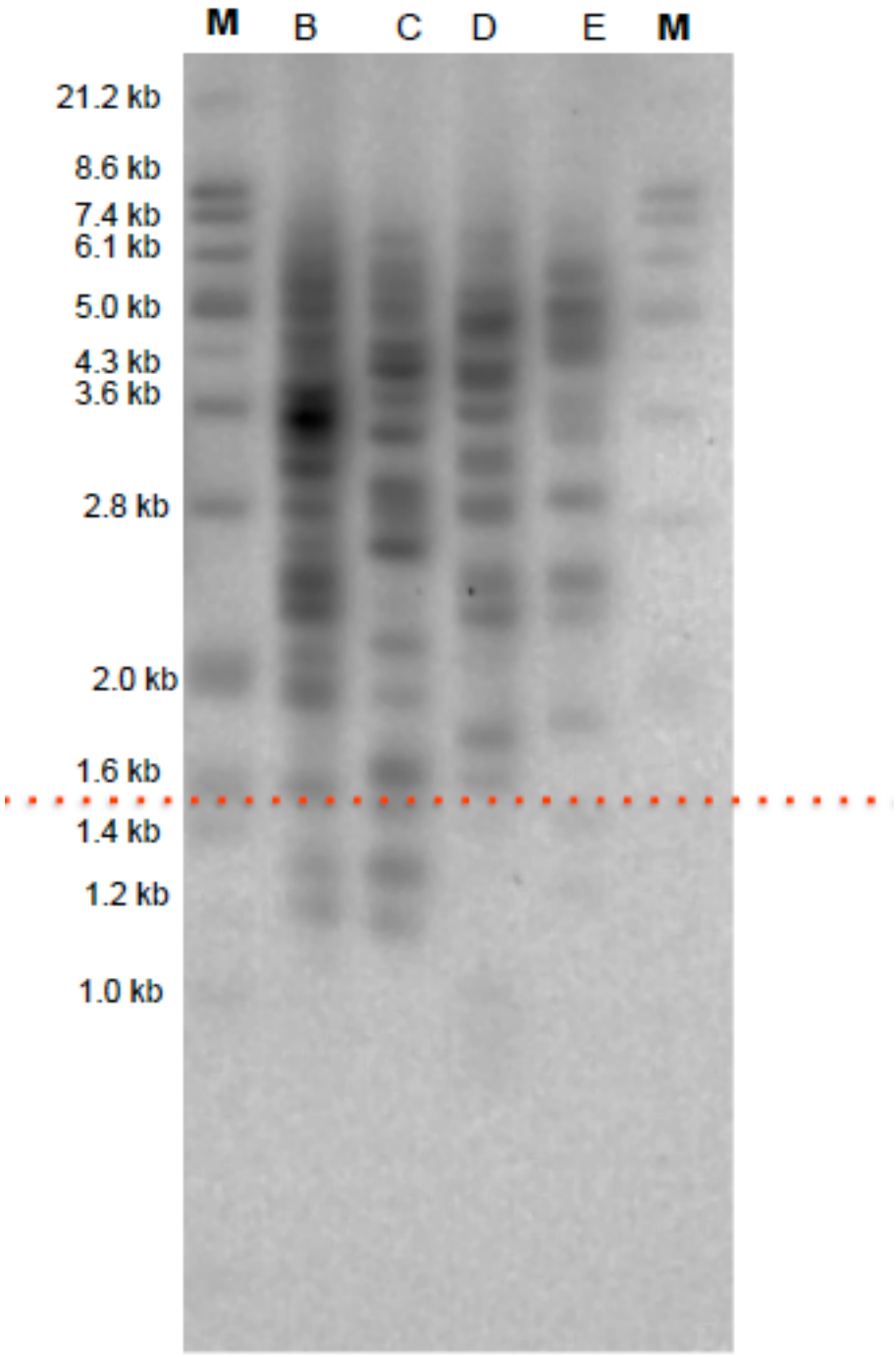

Figure 24(e): U-STELA Gel 1_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel1_1	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2335.619	2640.817	2540.360	2573.414	2475.521	2763.049	2246.772	2606.898	21226
2	8576	2039.161	2217.914	2232.297	2133.545	2133.545	2556.834	1760.335	2396.794	8576
3	7427	1936.900	1540.748	1961.898	1694.401	1694.401	2350.765	1559.893	2000	7427
4	6106	1630.937		1760.335	695.723	1164	1875.976	1341.609	1540.748	6106
5	5000	1451.207		1477.508			957.914		849.003	5000
6	4268	1138.895		1416.865						4268
7	3630	1059.057		1122.459						3630
8	2799	849.002		387.603						2799
9	2000	654.593								2000
10	1900	280.425								1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 25 (a): Telomere bands corresponding to Gel 1_1

Band Gel1_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2291.080	2374.485	2179.213	2568.817	2514.293	2496.376	2443.389	2258.545	21226
2	8576	1987.218	1887.562	1862.930	1924.521	2163.684	1974.517	2194.853	1875.206	8576
3	7427	1779.223	1814.627	1677.102	1756.005	1987.218	1756.005	1974.517	1633.617	7427
4	6106	1677.102	1245.519	1490.494	1502.209	1779.223	1688.153	1382.875	1292.366	6106
5	5000	1260.943	770.7533	889.191		1467.336	1525.918	1200.371	1185.688	5000
6	4268	1140.966				1032.457	1349.252		964.637	4268
7	3630	242.7649				854.769	1005.306		786.737	3630
8	2799						285.747			2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 25 (b): Telomere bands corresponding to Gel 1_2

Band Gel 1_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2540.360	2709.986	2844.850	2590.102	2623.803	2938.816	2821.832	2540.360	21226
2	8576	2290.765	2217.914	2459.572	2276.006	2305.620	2475.521	2459.572	2119.798	8576
3	7427	1900	2012.969	2246.772	1550	2133.545	2335.619	2246.772	1932.765	7427
4	6106	1334.572	1757.702	1877.381		1966.095	2147.379	1757.702	1747.208	6106
5	5000	1110.962	788.533	1171.986		1811.125	794.465	1514.0174	1635.827	5000
6	4268			584.656		862.761		998.631	1422.095	4268
7	3630			553.103		699.858		862.761	1103.585	3630
8	2799					606.069			1005.306	2799
9	2000								886.187	2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 25 (c): Telomere bands corresponding to Gel 1_3

Band Gel 1_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2519.918	2591.497	2416.238	2555.457	2702.697	2865.939	2467.534	2555.457	21226
2	8576	2014.054	2399.378	2042.459	2433.217	2349.499	2702.697	2300.658	2252.832	8576
3	7427	1855.030	1232.033	1520.699	1877.381	2115.236	2467.534	1855.030	1789.564	7427
4	6106	1454.469	1180.648	1473.092	1463.751	1757.702	1974.517	1747.208		6106
5	5000	1223.316		1189.060	619.457	1540.171	1800.312	916.032		5000
6	4268	714.671		957.914		809.488	1665.480			4268
7	3630			814.813			1597.110			3630
8	2799						1172.294			2799
9	2000						738.489			2000
10	1900						635.507			1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 25 (d): Telomere bands corresponding to Gel 1_4

Band Gel 1_5	A(M)	B	C	D	E	F(M)
1	21226	2388.624	2561.203	2799	2885.929	21226
2	8576	2284.906	2077.568	2388.624	2419.113	8576
3	7427	2051.383	1813.606	2241.846	2241.846	7427
4	6106	1867.130	1577.287	1711.116	1751.398	6106
5	5000	1559.042	1509.034	1568.138	1461.302	5000
6	4268	1369.020	1338.726	1017.363	1294.537	4268
7	3630	1244.823	1217.277			3630
8	2799					2799
9	2000					2000
10	1900					1900
11	1550					1550
12	1400					1400
13	1164					1164
14	992					992
15	925					925
16	831					831
17	710					710
18	564					564
19	492					492
20	359					359

Table 25 (e): Telomere bands corresponding to Gel 1_5

GEL 2

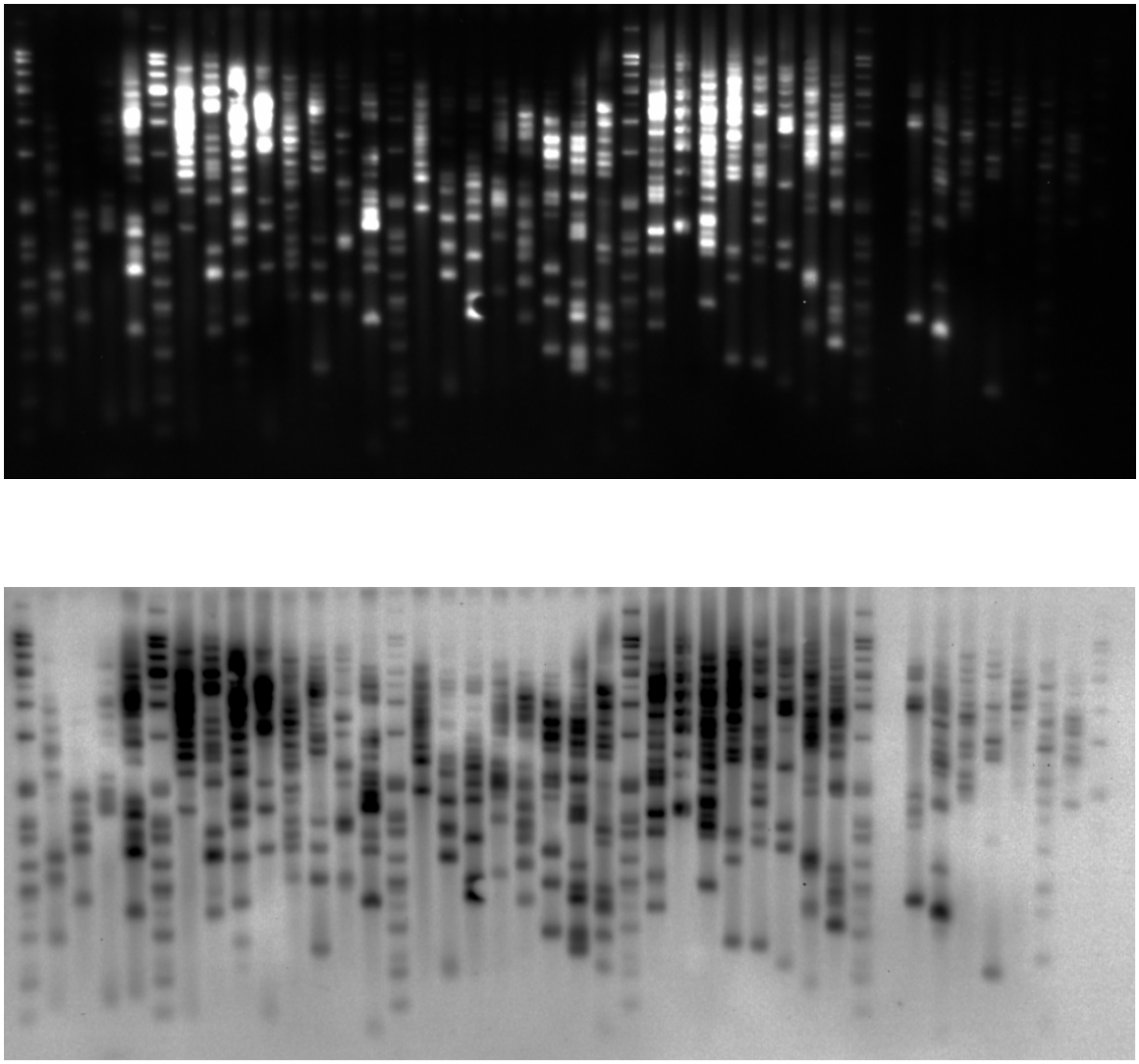

Figure 25: U-STELA Gel 2

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 2_1

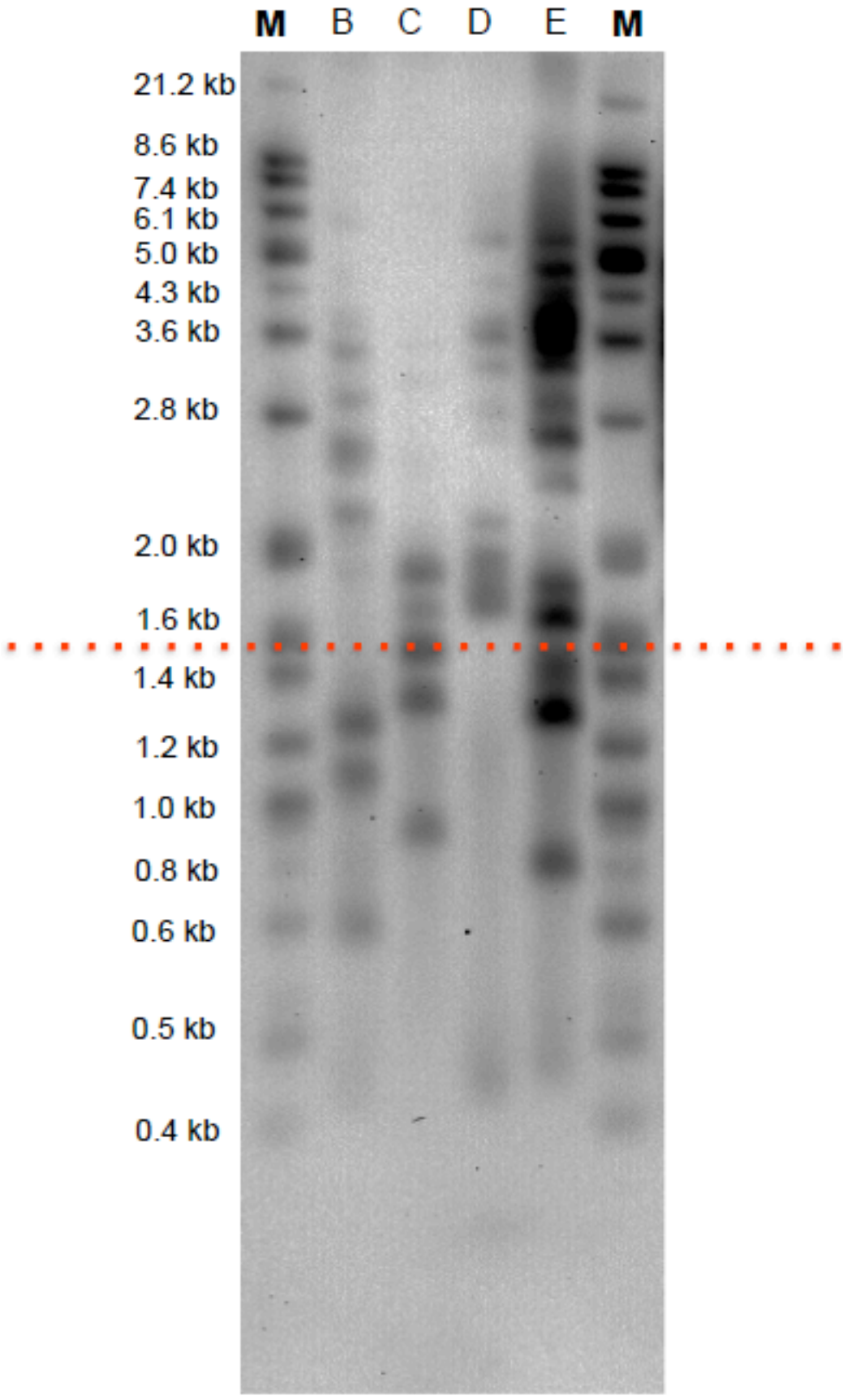

Figure 25 (a): U-STELA Gel 2_1 with markers

GEL 2_2

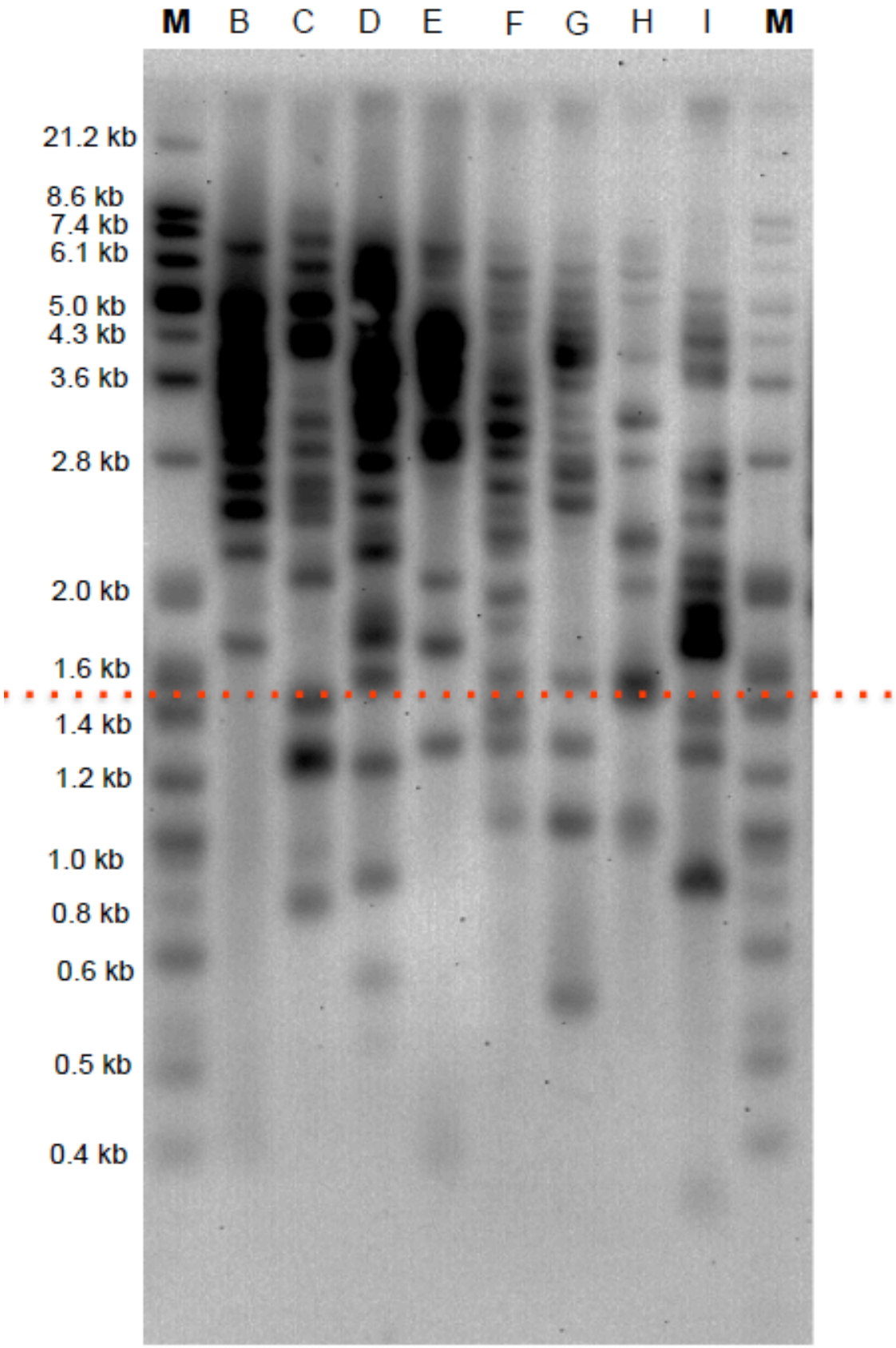

Figure 25 (b): U-STELA Gel 2_2 with markers

GEL 2_3

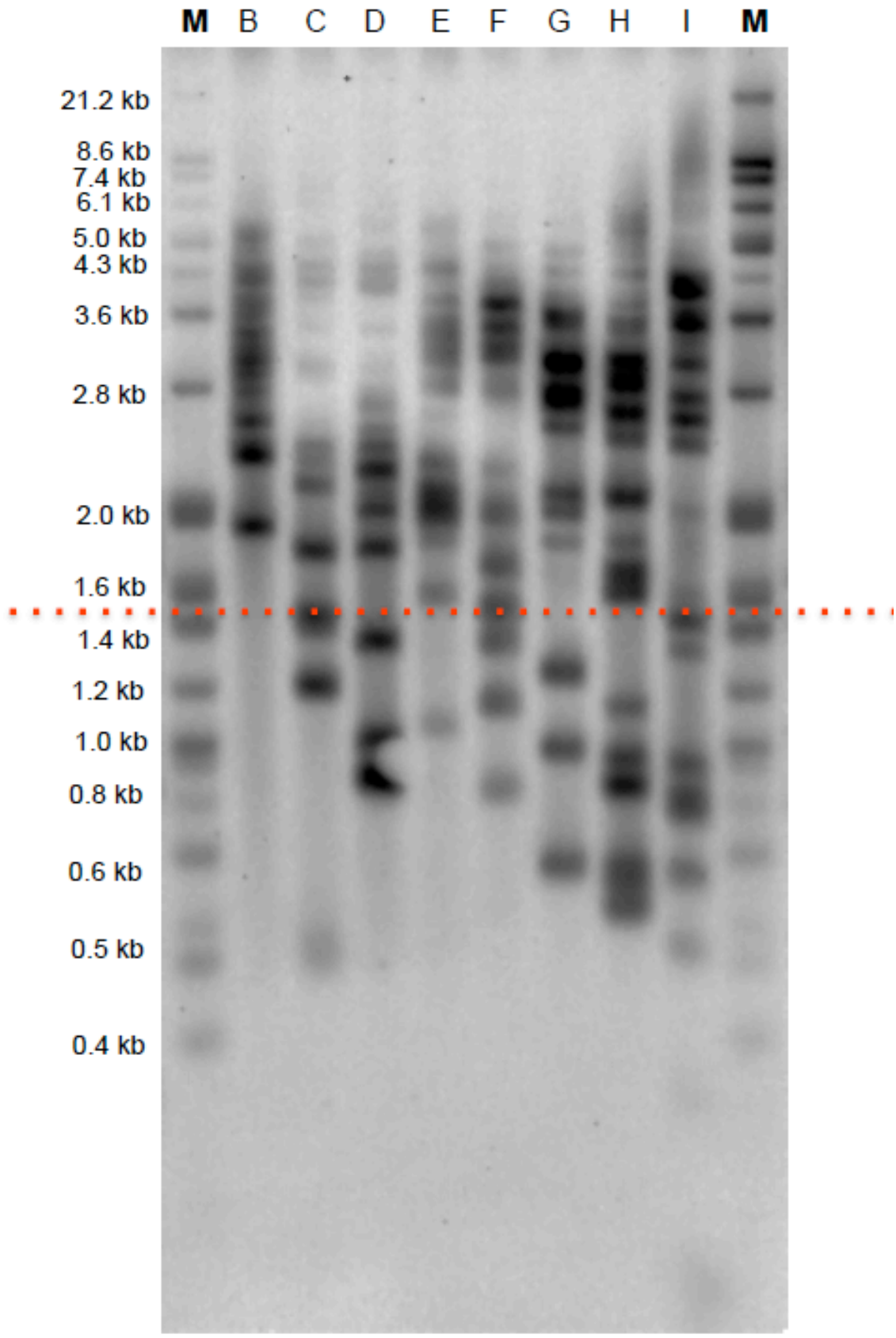

Figure 25 (c): U-STELA Gel 2_3 with markers

GEL 2_4

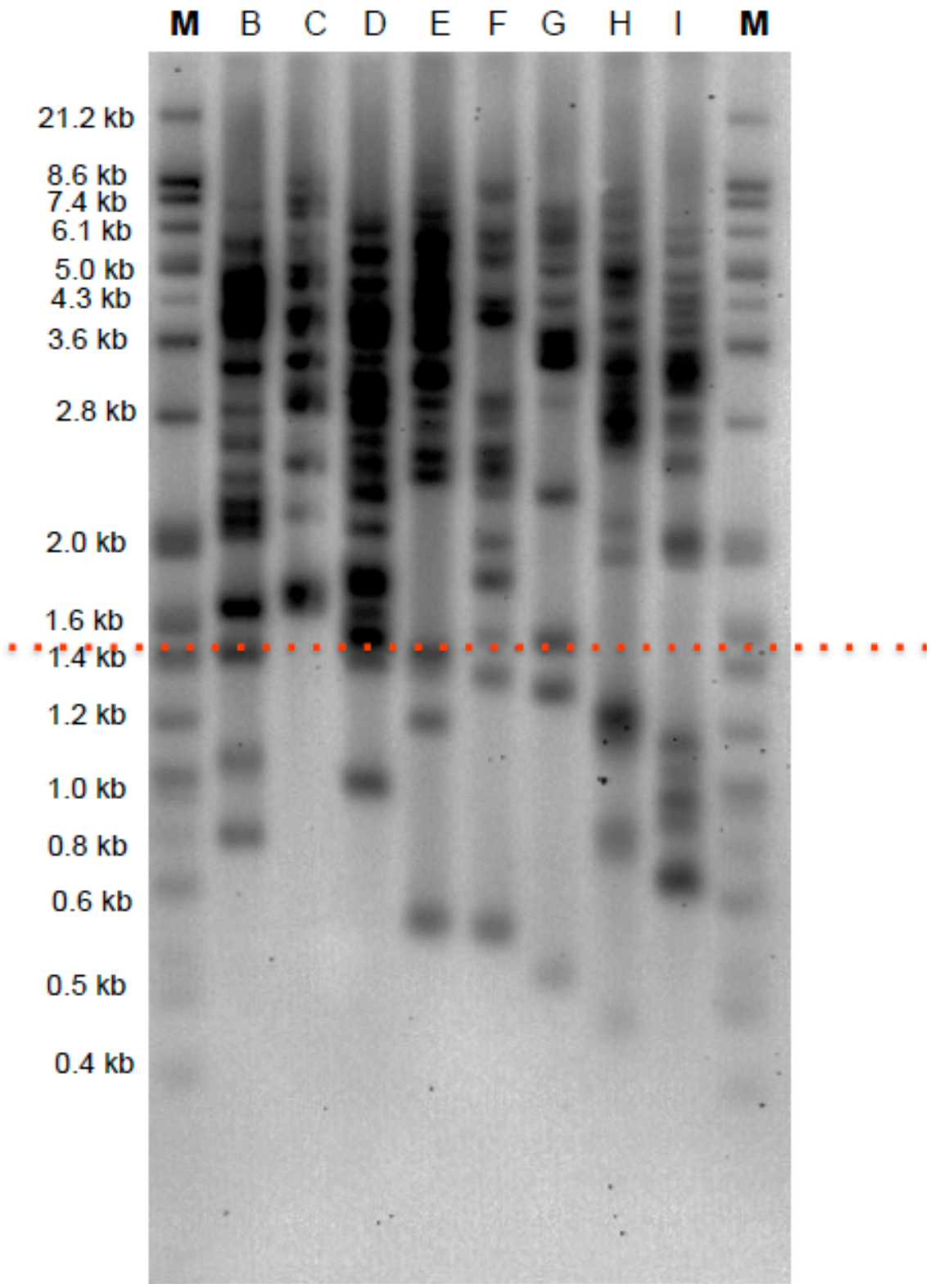

Figure 25 (d): U-STELA Gel 2_4 with markers

GEL 2_5

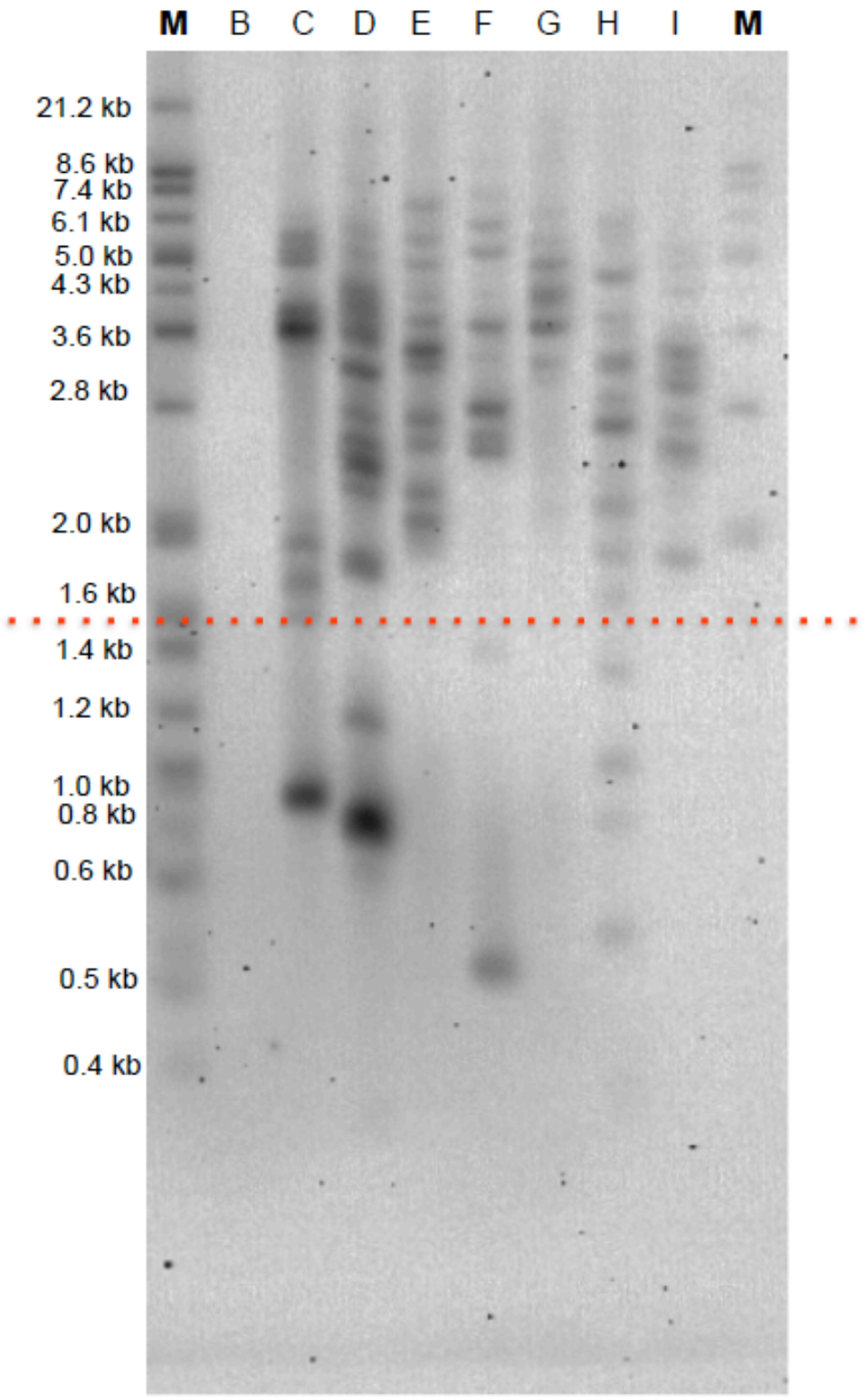

Figure 25 (e): U-STELA Gel 2_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 2_1	A(M)	B	C	D	E	F(M)
1	21226	1866.172	1877.381	1959.383	1789.564	21226
2	8576	1242.802	1705.855	1757.702	1655.537	8576
3	7427	1084.152	1504.287	1213.548	1400	7427
4	6106	705.474	1334.866	423.967	1288.012	6106
5	5000	405.809	925		852.220	5000
6	4268				500.472	4268
7	3630				446.831	3630
8	2799					2799
9	2000					2000
10	1900					1900
11	1550					1550
12	1400					1400
13	1164					1164
14	992					992
15	925					925
16	831					831
17	710					710
18	564					564
19	492					492
20	359					359

Table 26 (a): Telomere bands corresponding to Gel 2_1

Band Gel 2_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1695.670	1822.003	1832.946	2055.867	1949.359	1529.107	2022.162	2022.162	21226
2	8576	371.793	1428.791	1726.407	1695.671	1800.312	1287.315	1529.107	1855.030	8576
3	7427		1230.973	1550	1280.134	1550	1054.026	1210.486	1675.483	7427
4	6106		969.144	1217.277	343.625	1392.189	639.078	1048.231	1376.700	6106
5	5000		825.986	876.741		1287.315			1258.829	5000
6	4268			673.606		1059.854			886.187	4268
7	3630			535.842					301.339	3630
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 26 (b): Telomere bands corresponding to Gel 2_2

Band Gel 2_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1870.475	1766.088	2000	2037.005	1959.383	2087.413	1793.966	1959.383	21226
2	8576		1473.092	1775.332	1831.821	1702.714	2000	1650.207	1803.355	8576
3	7427		1186.443	1356.141	1582.707	1516.559	1803.355	1599.319	1566.268	7427
4	6106		507.754	1027.882	1058.772	1364.801	1658.844	1116.734	1451.828	6106
5	5000			881.022		1130.039	1240.508	935.844	1322.045	5000
6	4268					864.022	992	872.481	916.032	4268
7	3630						692.069	666.019	839.135	3630
8	2799							616.822	678.919	2799
9	2000								518.535	2000
10	1900								275.318	1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 26 (c): Telomere bands corresponding to Gel 2_3

Band Gel 2_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1609.237	1688.737	1772.164	1419.129	1932.765	1478.098	2084.282	1932.765	21226
2	8576	1419.129		1697.809	1157.127	1743.907	1268.169	1849.776	1084.152	8576
3	7427	1033.987		1600.638	634.888	1488.162	526.771	1164	1003.819	7427
4	6106	831		1488.162		1319.342		825.569	909.480	6106
5	5000			1409.532		626.591		434.447	864.465	5000
6	4268			969.144					724.104	4268
7	3630									3630
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 26 (d): Telomere bands corresponding to Gel 2_4

Band Gel 2_5	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226		1869.704	1781.685	2000	1400		1820.281	1800.879	21226
2	8576		1725.319	1137.713	1869.704	526.771		1617.882		8576
3	7427		1550	844.466				1300.343		7427
4	6106		905.386	737.328				1014.920		6106
5	5000							849.003		5000
6	4268							590.575		4268
7	3630									3630
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 26 (e): Telomere bands corresponding to Gel 2_5

GEL 3

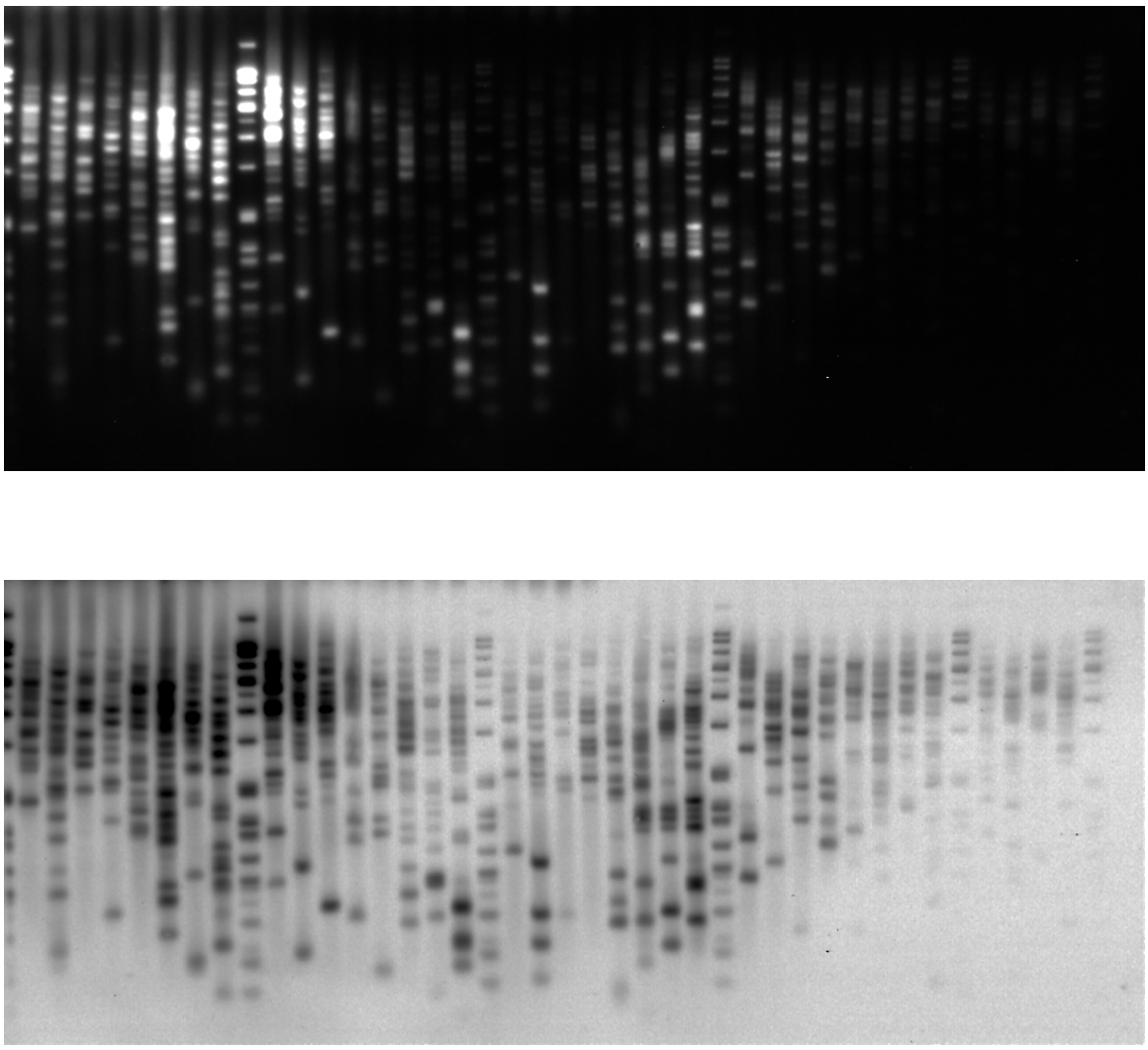

Figure 26: U-STELA Gel 3

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 3_1

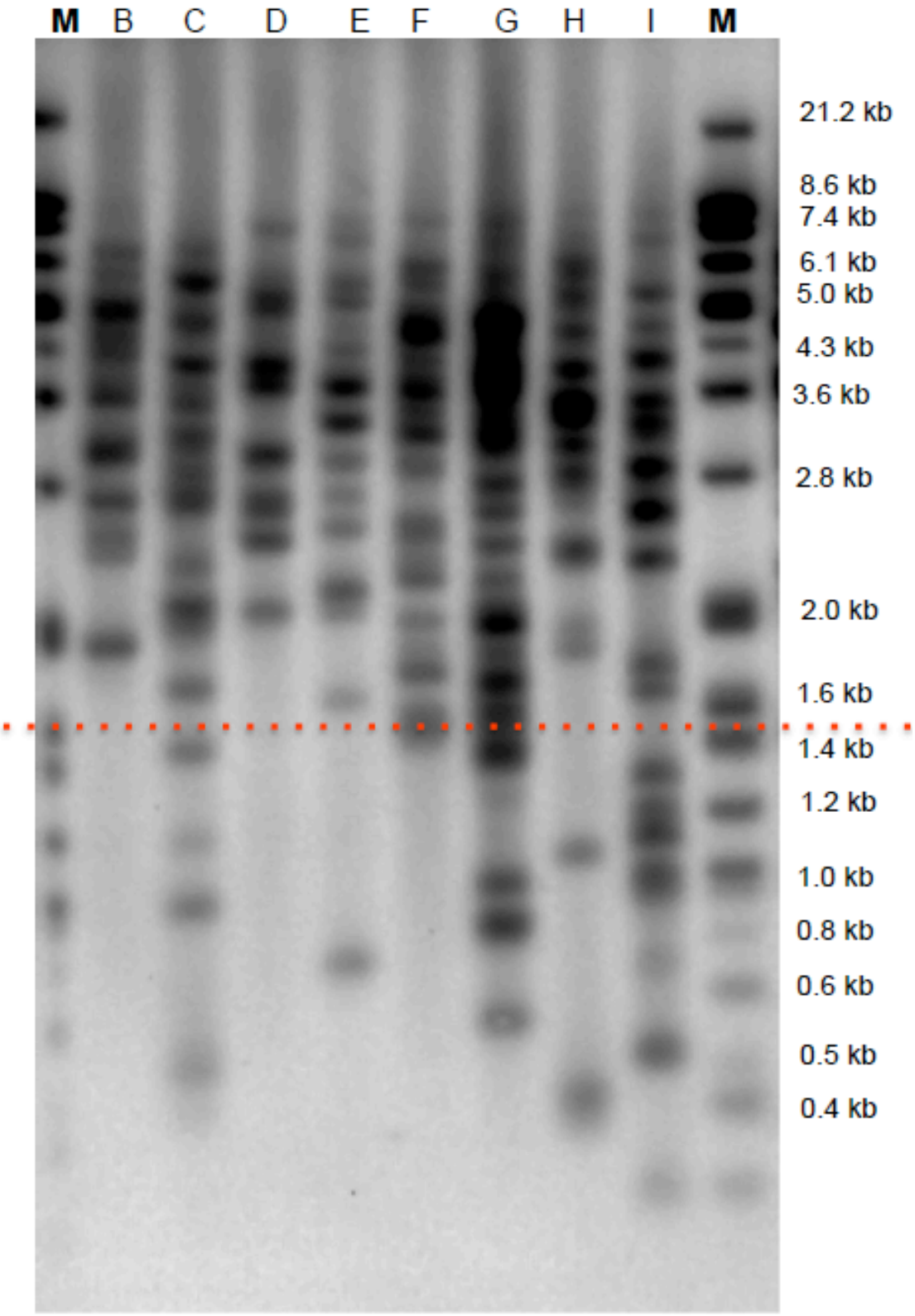

Figure 26 (a): U-STELA Gel 3_1 with markers

GEL 3_2

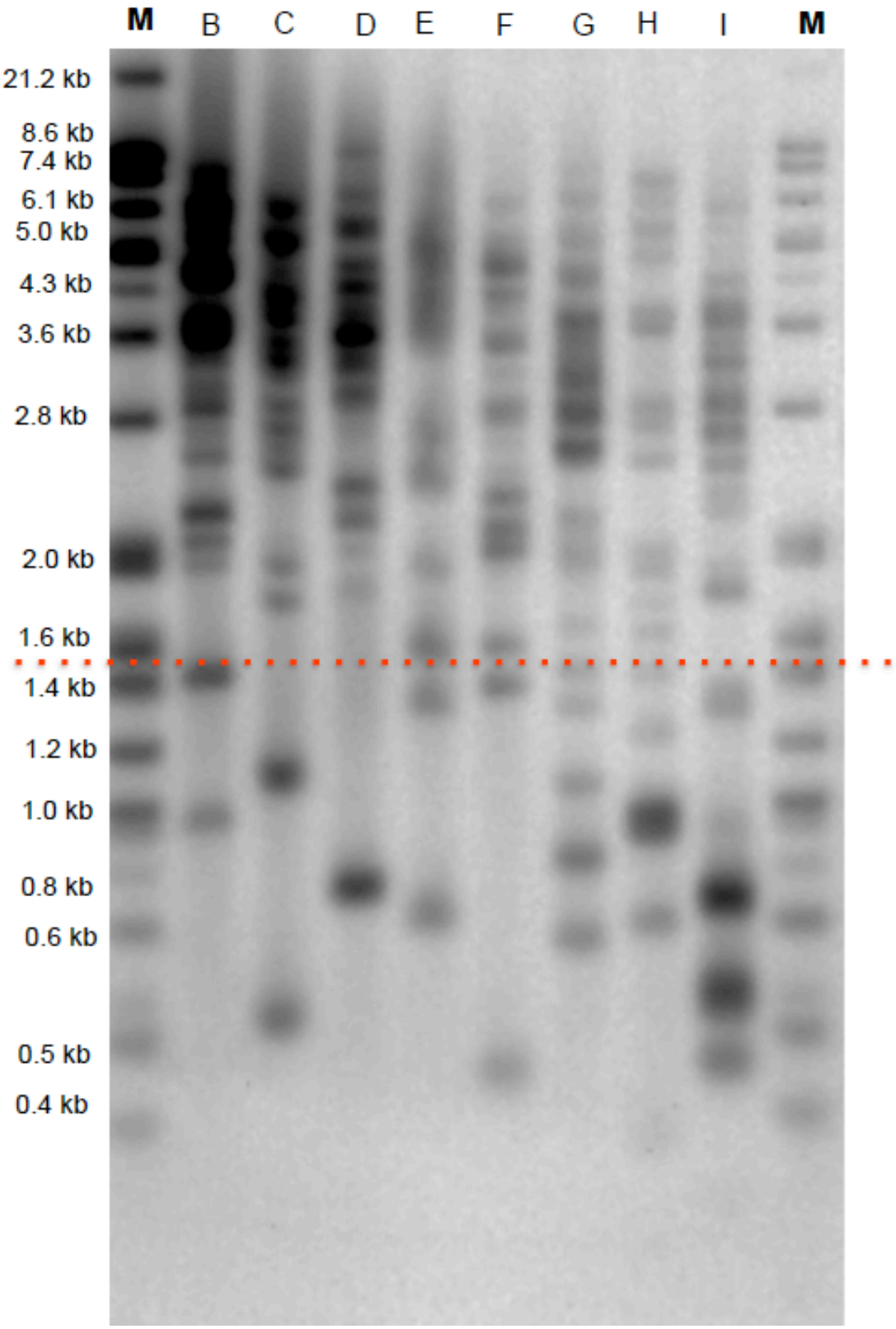

Figure 26 (b): U-STELA Gel 3_2 with markers

GEL 3_3

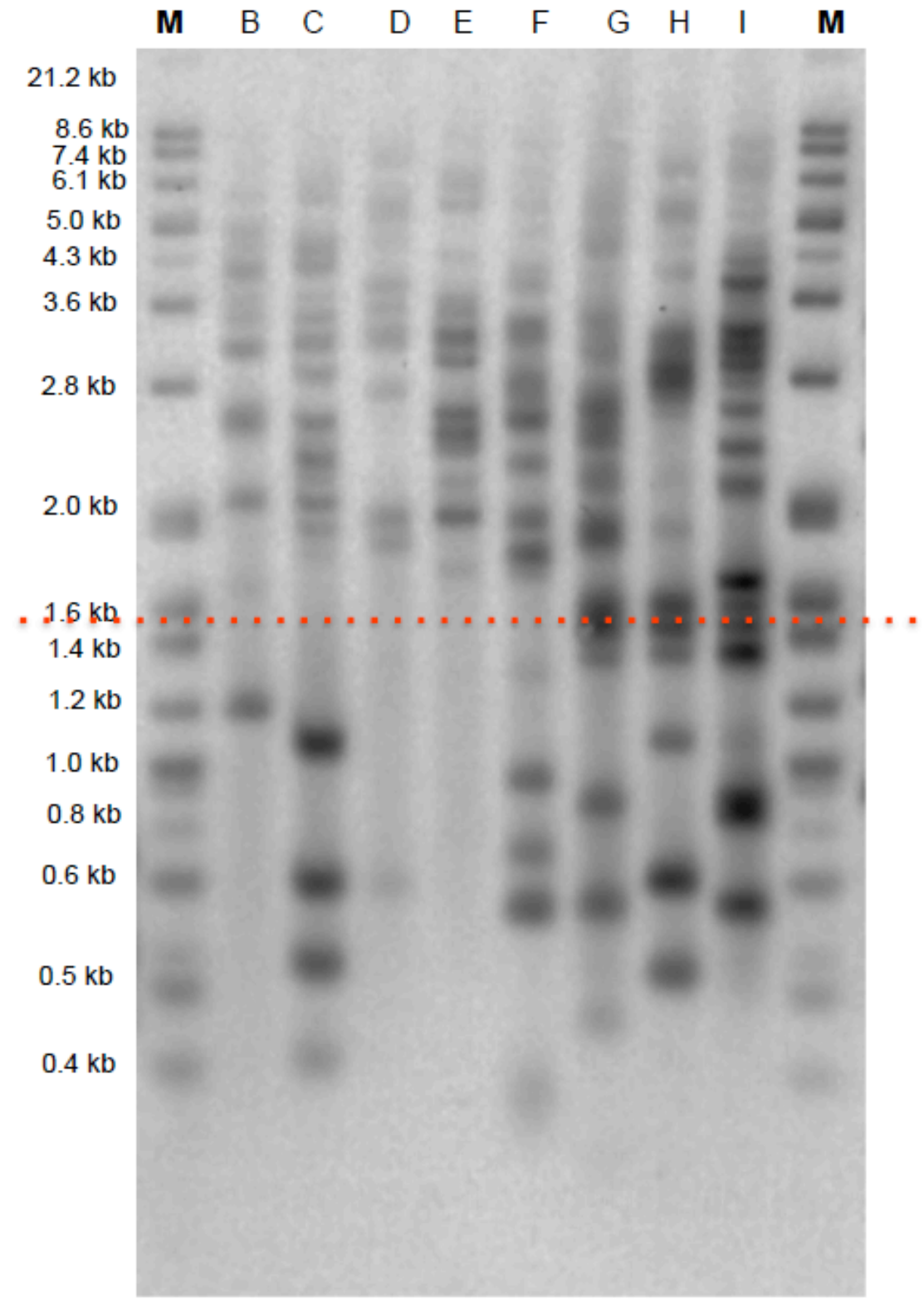

Figure 26 (c): U-STELA Gel 3_3 with markers

GEL 3_4 and GEL 3_5

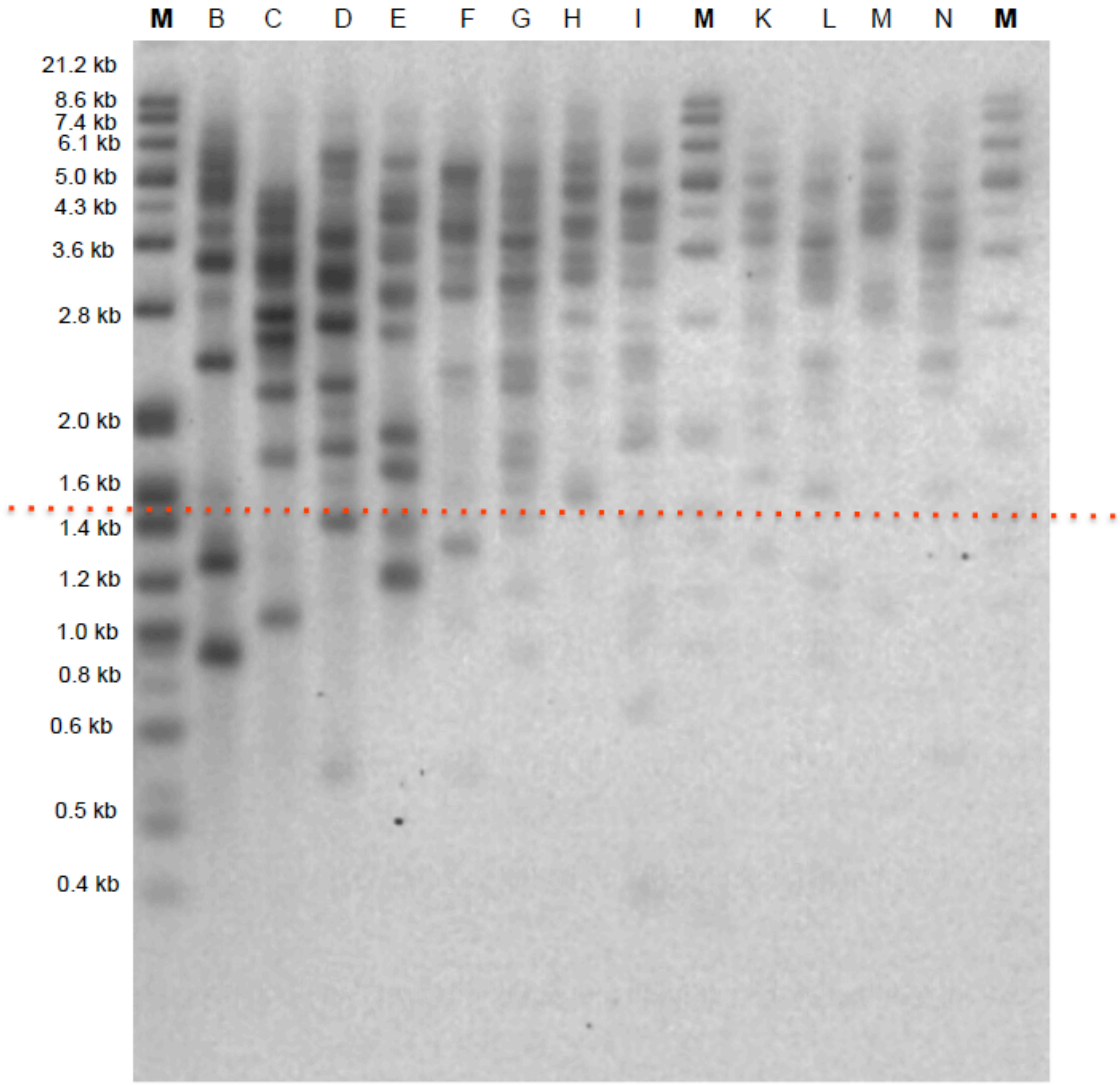

Figure 26 (d): U-STELA Gel 3_4 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 3_1	A	B	C	D	E	F	G	H	I	J
1	21226	2633.069	2781.947	2601.083	2930.401	2885.929	2731.409	2820.484	2842.132	21226
2	8576	2387.798	2633.069	2387.798	2649.210	2446.886	2569.485	2330.136	2569.485	8576
3	7427	2315.939	2218.957	2000	2461.885	2178.646	2344.419	1855.030	2287.806	7427
4	6106	1789.564	2024.595		2100.209	1900	2165.373	1778.879	1705.855	6106
5	5000		1606.703		2000	1695.670	1888.656	1037.421	1616.353	5000
6	4268		1356.141		1568.675	1455.893	1726.407	501.694	1280.628	4268
7	3630		1071.133		760.278		1635.827		1149.205	3630
8	2799		874.272				1514.017		1091.884	2799
9	2000		564				1356.140		974.808	2000
10	1900						1201.645		760.278	1900
11	1550						919.797		593.129	1550
12	1400						840.427		359	1400
13	1164						660.716			1164
14	992						637.372			992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 27 (a): Telomere bands corresponding to Gel 3_1

Band Gel 3_2	A	B	C	D	E	F	G	H	I	A
1	21226	2074.695	1924.521	2037.005	1900	2049.492	2165.373	2012.259	1900	21226
2	8576	1949.359	1742.775	1797.374	1550	1559.593	2000	1888.314	1786.319	8576
3	7427	1441.311	1105.940	803.052	1363.561	1400	1638.501	1742.775	1328.069	7427
4	6106	980.505	543.829		749.946	455.811	1462.421	1608.453	969.144	6106
5	5000						1319.342	1441.311	781.372	5000
6	4268						1071.133	1243.331	588.878	4268
7	3630						863.033	980.505	469.059	3630
8	2799						694.841	734.709		2799
9	2000							348.860		2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 27 (b): Telomere bands corresponding to Gel 3_2

Band Gel 3_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1645.653	1924.521	2012.259	2024.595	2000	1900	1924.521	1675.483	21226
2	8576	1171.986	1064.304	1832.946	1726.407	1789.564	1550	1568.675	1578.097	8576
3	7427		710	704.747		1289.718	1502.209	1478.870	1490.494	7427
4	6106		553.103			957.914	1352.947	1371.575	1371.575	6106
5	5000		376.558			776.045	888.565	1071.133	1057.519	5000
6	4268					649.466	659.184	714.874	870.889	4268
7	3630					323.199		531.935	664.098	3630
8	2799								553.103	2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 27 (c): Telomere bands corresponding to Gel 3_3

Band Gel 3_4.5	A(M)	B	C	D	E	F	G	H	I
1	21226	1550	1736.777	2037.005	1855.030	2205.438	1811.125	2246.244	2358.791
2	8576	1332.748	1260.943	1789.564	1675.483	1587.575	1716.100	1559.309	1822.003
3	7427	1237.878	1044.078	1616.353	1391.411	1300.343	1578.097		1391.411
4	6106	914.051		1400	1185.688	1030.808	1391.411		992
5	5000			610.446		606.069	1134.599		765.498
6	4268						903.232		365.717
7	3630								
8	2799								
9	2000								
10	1900								
11	1550								
12	1400								
13	1164								
14	992								
15	925								
16	831								
17	710								
18	564								
19	492								
20	359								

Table 27 (d): Telomere bands corresponding to Gel 3_4

BAND Gel 3_4.5	J(M)	K	L	M	N	O(M)
1	21226	2087.413	1578.097	1078.006	1587.575	21226
2	8576	1888.656	1178.414		632.803	8576
3	7427	1655.537	887.243			7427
4	6106	1260.943				6106
5	5000					5000
6	4268					4268
7	3630					3630
8	2799					2799
9	2000					2000
10	1900					1900
11	1550					1550
12	1400					1400
13	1164					1164
14	992					992
15	925					925
16	831					831
17	710					710
18	564					564
19	492					492
20	359					359

Table 27 (e): Telomere bands corresponding to Gel 3_5

GEL 4

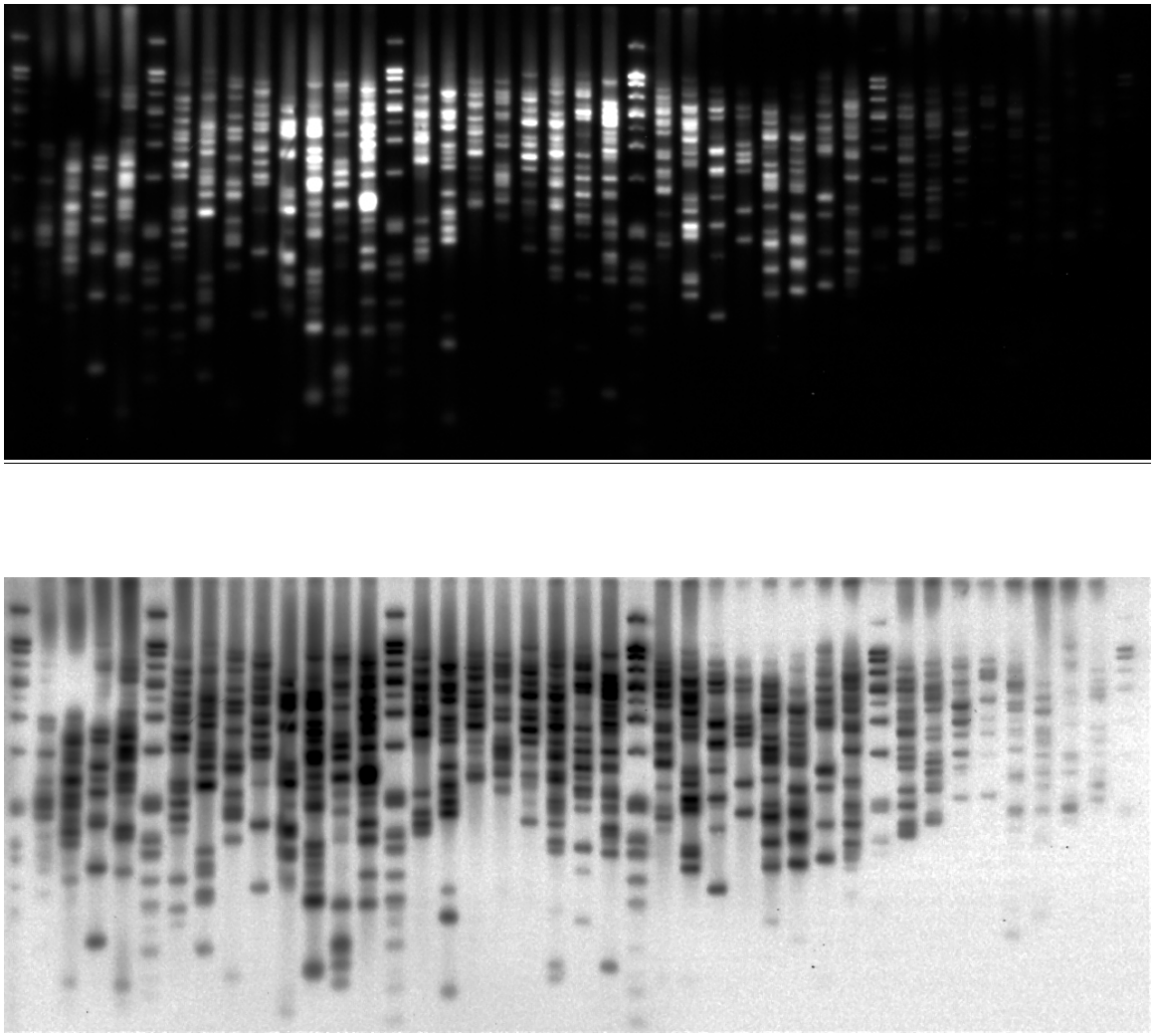

Figure 27: U-STELA Gel 4

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 4_1

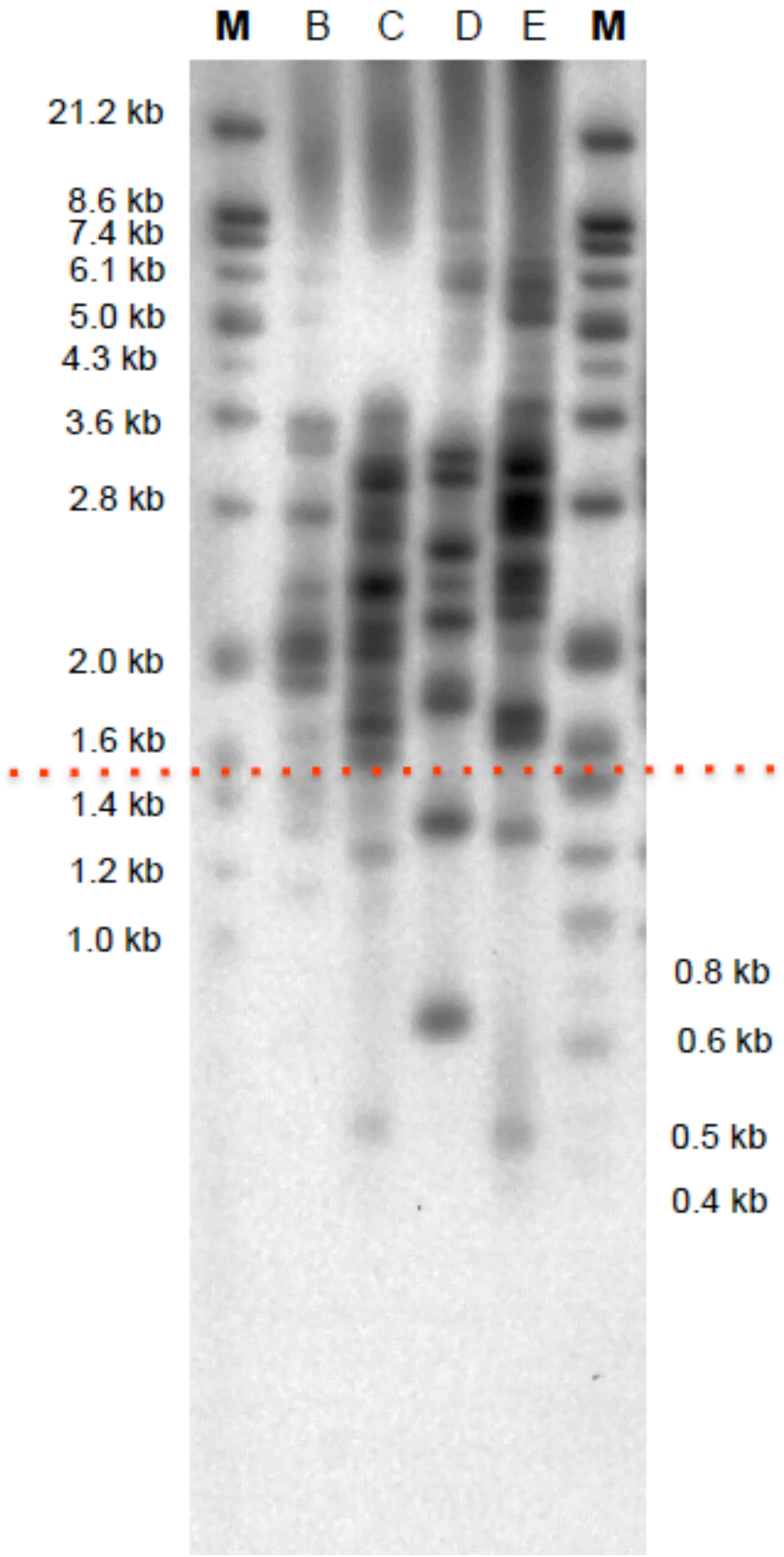

Figure 27 (a): U-STELA Gel 4_1 with markers

GEL 4_2

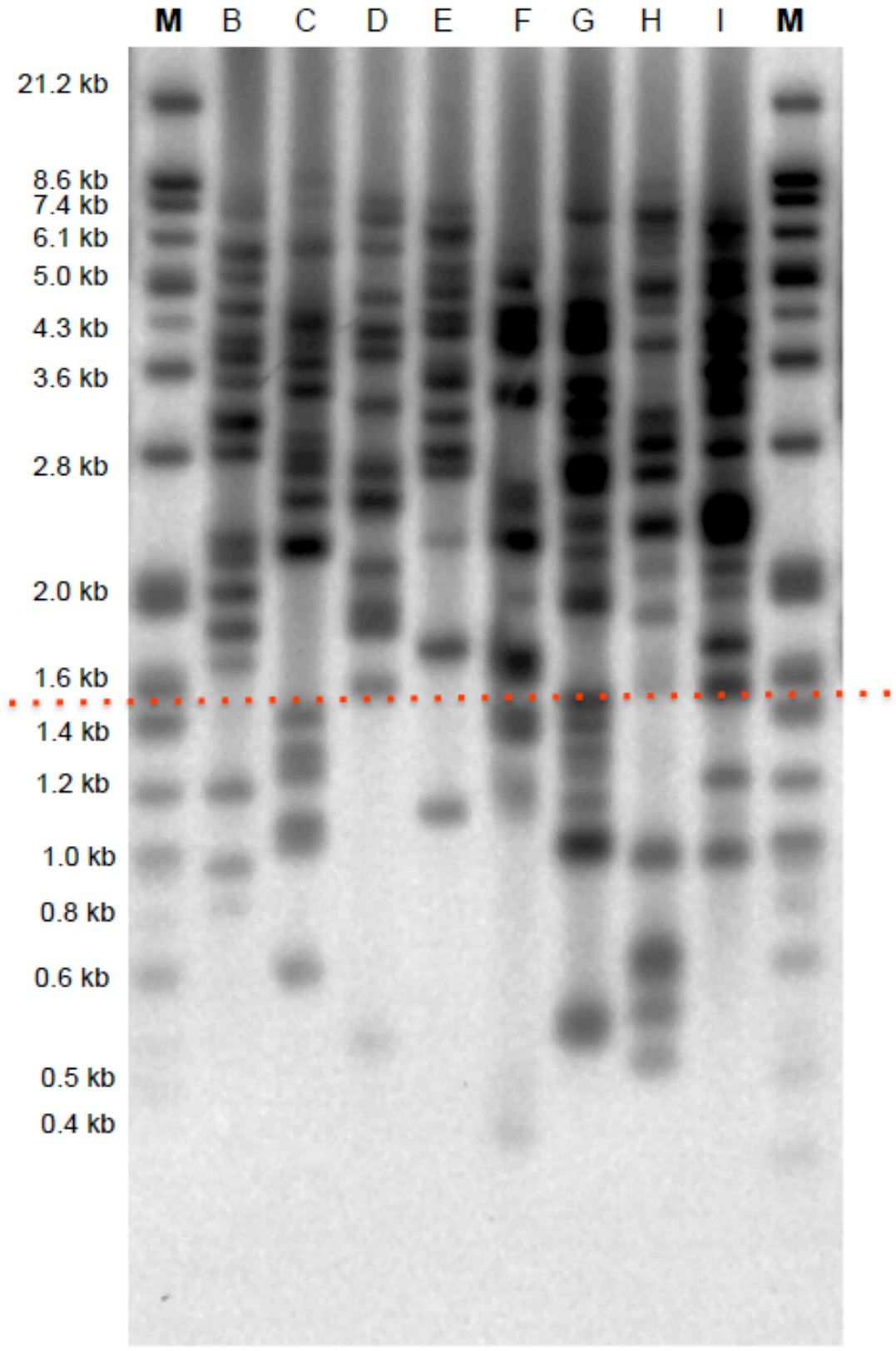

Figure 27 (b): U-STELA Gel 4_2 with markers

GEL 4_3

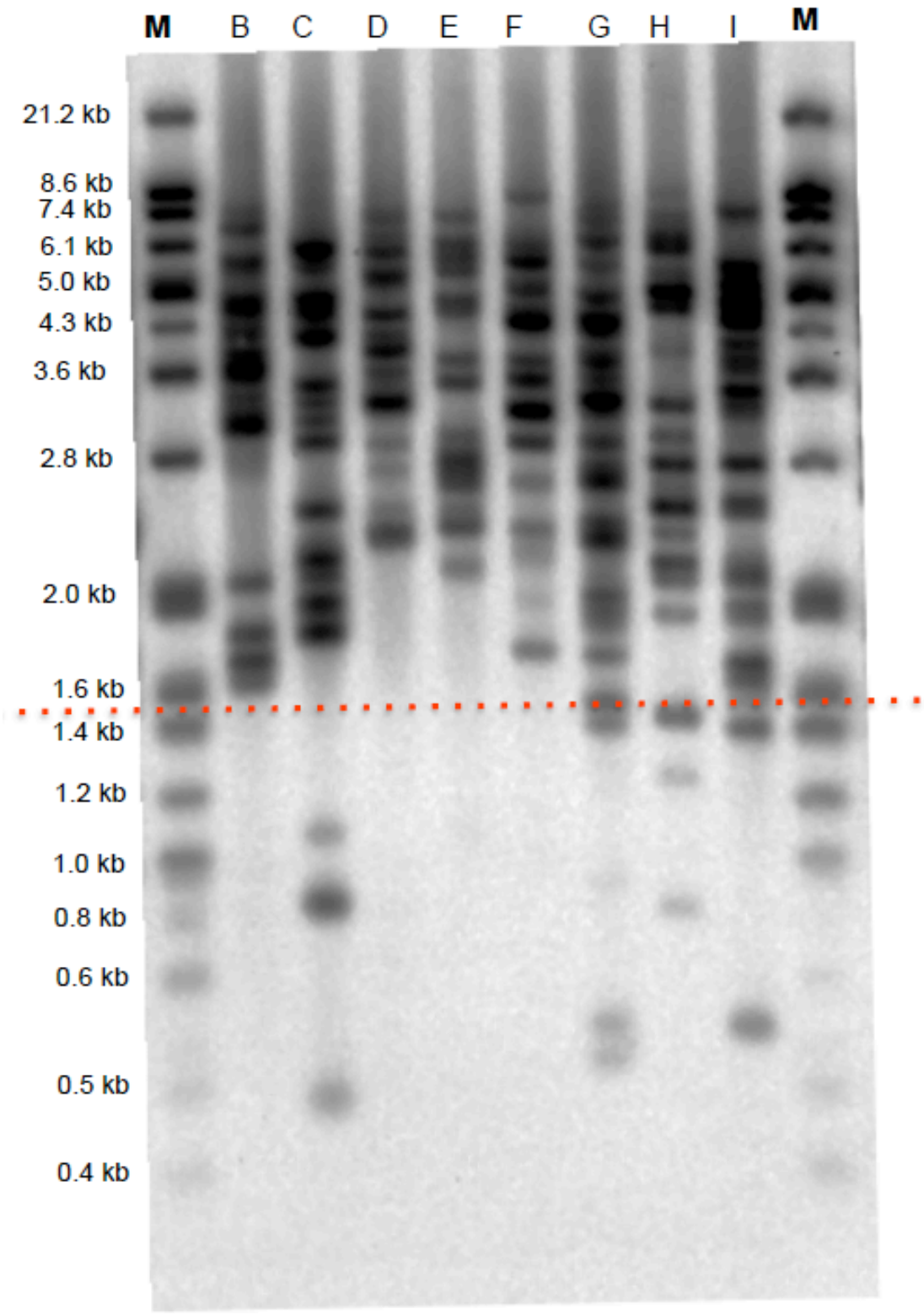

Figure 27 (c): U-STELA Gel 4_3 with markers

GEL 4_4

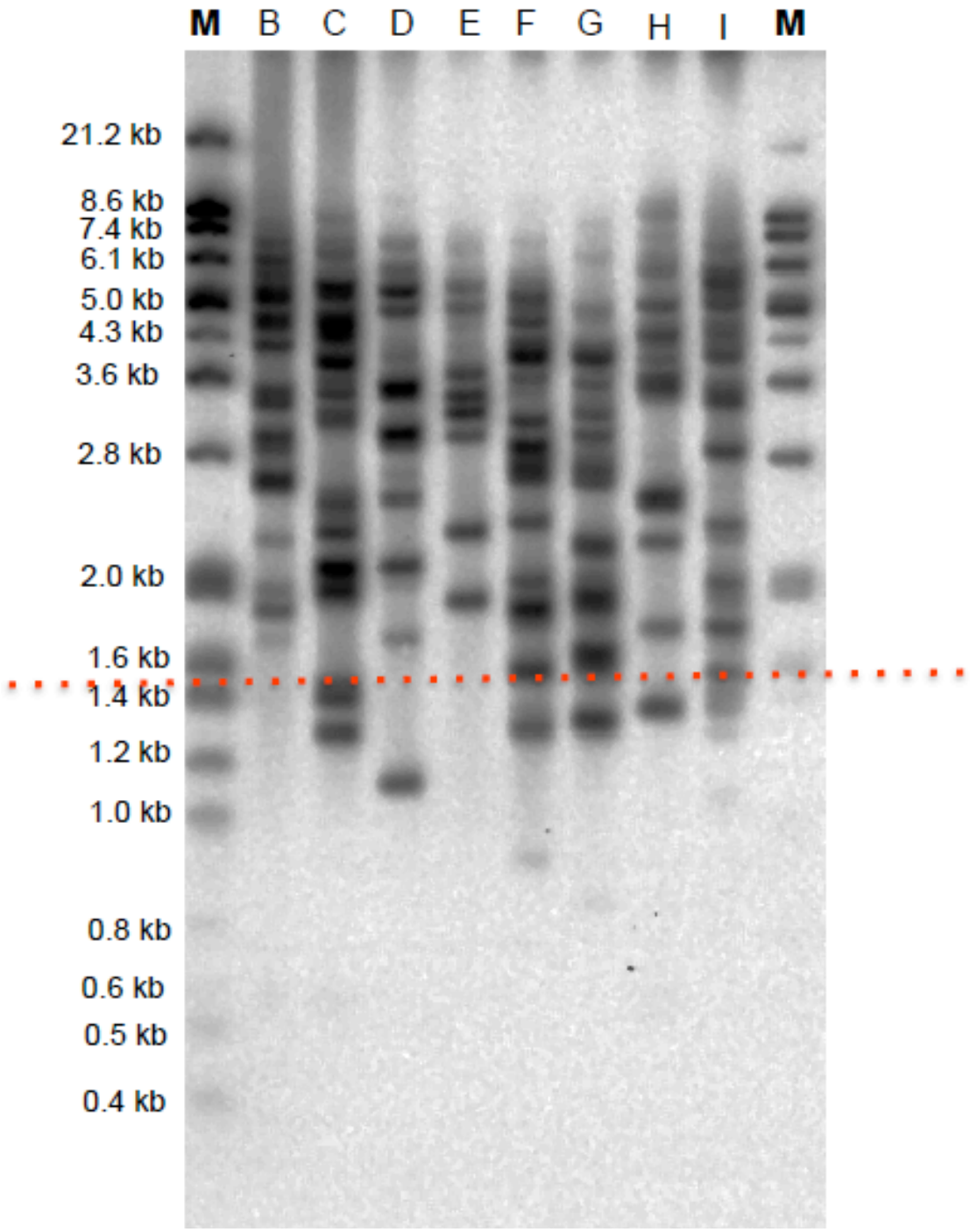

Figure 27 (d): U-STELA Gel 4_4 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 4_1	A(M)	B	C	D	E	F(M)
1	21226	6491.379	3686.409	9045.587	6652.258	21226
2	8576	5214.839	2998.397	6106	6042.116	8576
3	7427	3658.096	2734.864	3261.496	5382.005	7427
4	6106	3311.755	2610.968	3044.602	4598.067	6106
5	5000	2734.864	2298.437	2507.172	4170.412	5000
6	4268	2285.156	2070.764	2338.745	3772.671	4268
7	3630	2011.624	1924.521	2131.643	3115.248	3630
8	2799	1798.274	1730.324	1701.994	2820.484	2799
9	2000	1575.799	1619.757	1276.558	2656.758	2000
10	1900	1438.006	1525.289	770.753	2379.760	1900
11	1550	1276.558	1164		2169.026	1550
12	1400	1080.718	534.015		2011.623	1400
13	1164				1655.804	1164
14	992				1593.238	992
15	925				1258.558	925
16	831				630.111	831
17	710				526.771	710
18	564					564
19	492					492
20	359					359

Table 28 (a): Telomere bands corresponding to Gel 4_1

Band Gel 4_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2986.956	2667.778	2604.492	2749.052	2408.998	2573.414	2604.492	2452.768	21226
2	8576	2749.052	2438.090	2423.500	2620.171	2175.320	2296.0596	2296.0596	2255.086	8576
3	7427	2175.320	2162.303	2048.597	2188.416	1900	2123.717	2048.597	2048.597	7427
4	6106	2098.376	1373.517	1789.564	1655.537	1616.353	1866.172	1811.125	1924.521	6106
5	5000	1912.222	1240.508	1494.668	1068.445	1347.535	1420.505	1505.574	1675.483	5000
6	4268	1736.777	1171.433	550.569		1118.388	1338.984	925	1494.668	4268
7	3630	1616.353	1032.457			398.766	1240.508	714.874	1171.433	3630
8	2799	1137.713	964.637				1093.132	595.124	925	2799
9	2000	898.357	678.051				978.223	516.297		2000
10	1900	814.117					590.575			1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 28 (b): Telomere bands corresponding to Gel 4_2

Band Gel 4_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2855.537	3011.948	3011.948	3052.372	3011.948	2991.938	3072.787	2836.566	21226
2	8576	2062.055	2522.807	2799	2855.537	2698.228	2714.767	2836.566	2538.271	8576
3	7427	1765.320	2218.957	2522.807	2417.161	2387.798	2387.798	2538.271	2126.036	7427
4	6106	1658.844	2139.068	2358.791	2152.180	2260.014	2330.136	2373.249	1878.630	6106
5	5000		1900		1046.306	1949.359	2024.595	2192.001	1649.489	5000
6	4268		1775.332			1706.422	1815.952	2113.083	1567.632	4268
7	3630		1039.359				1677.714	1847.025	1391.116	3630
8	2799		851.488				1501.475	1445.246	586.060	2799
9	2000		482.963				1417.926	1209.319		2000
10	1900						889.647	843.233		1900
11	1550						591.709			1550
12	1400						528.338			1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 28 (c): Telomere bands corresponding to Gel 4_3

Band Gel 4_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2919.521	3154.115	2960.837	2960.837	3110.102	3176.355	2483.399	2799	21226
2	8576	2599.198	2455.264	2628.982	2279.999	2838.610	2899.079	2228.631	2319.301	8576
3	7427	2241.364	2267.047	2483.399	1855.030	2644.002	2628.982	1716.100	1979.588	7427
4	6106	1919.592	2081.367	2081.368		2359.279	2190.866	1364.80	1716.100	6106
5	5000	1811.125	1939.386	1665.480		2000	1866.172	606.863	1518.766	5000
6	4268	1665.480	1448.314	1081.747		1822.003	1587.575		1356.140	4268
7	3630	626.216	1400			1518.766	1313.655		1067.429	3630
8	2799		1264.426			1288.806	846.983			2799
9	2000		652.984			890.418				2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	925									925
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 28 (d): Telomere bands corresponding to Gel 4_4

GEL 5

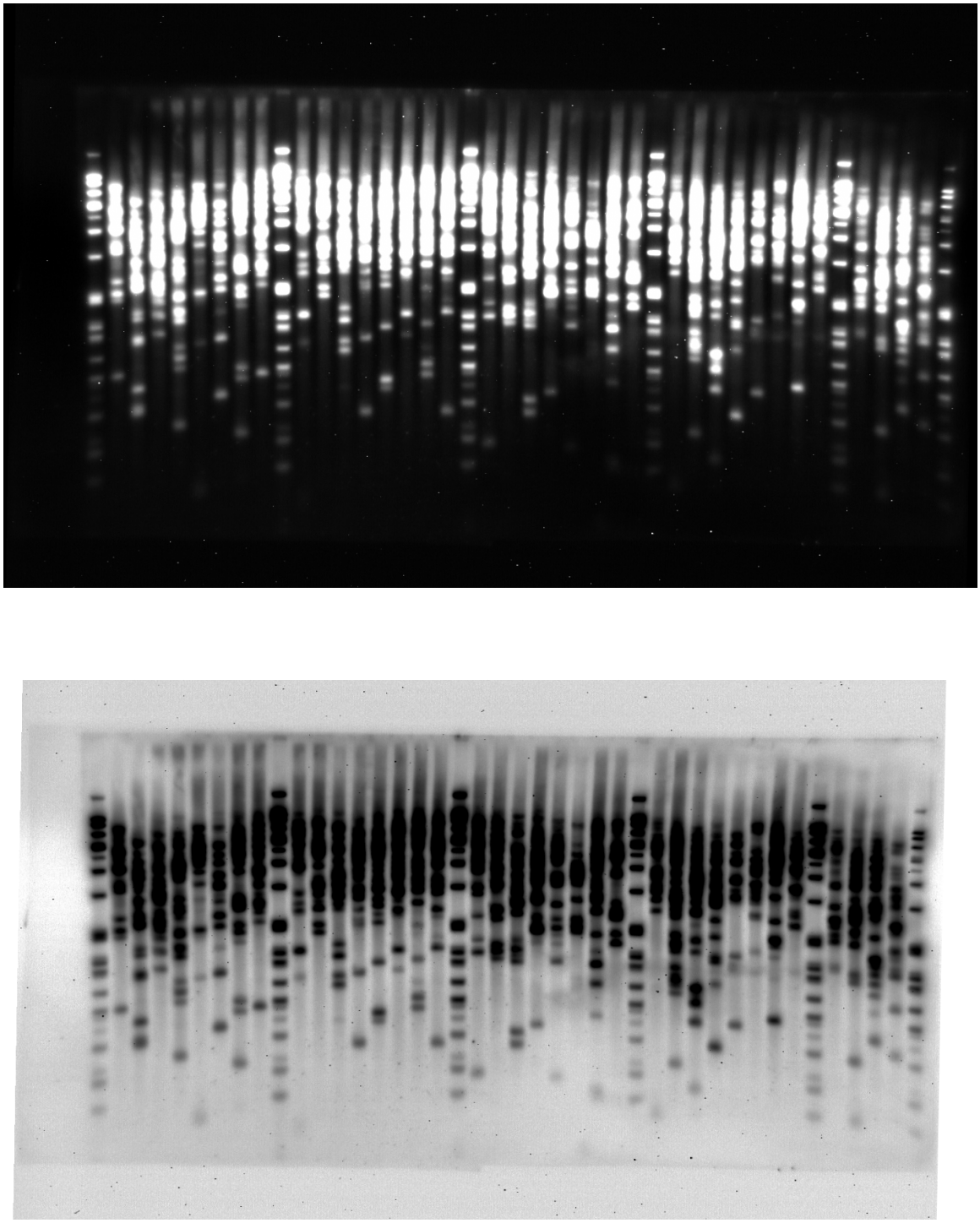

Figure 28: U-STELA Gel 5

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 5_1

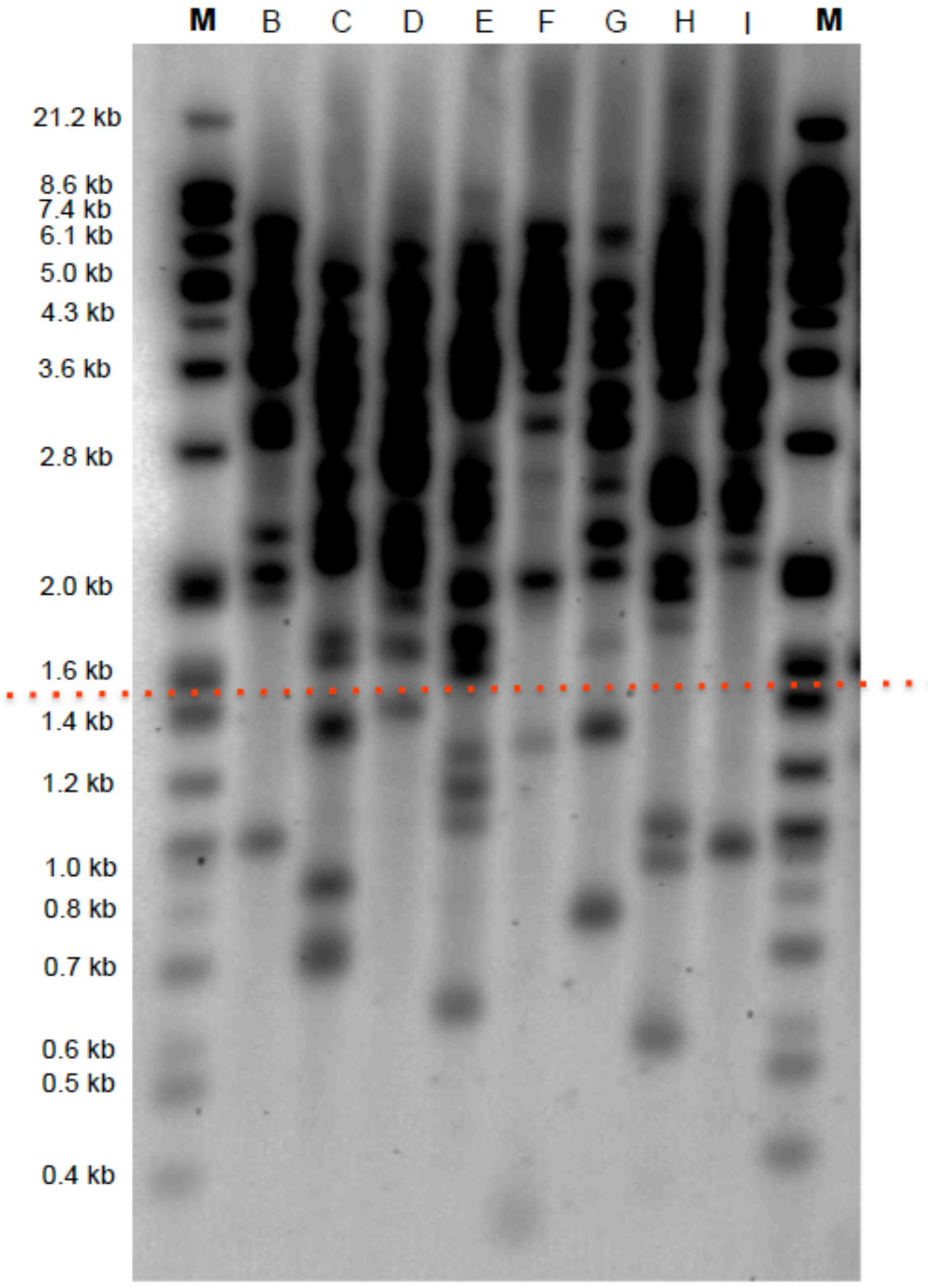

Figure 28 (a): U-STELA Gel 5_1 with markers

GEL 5_2

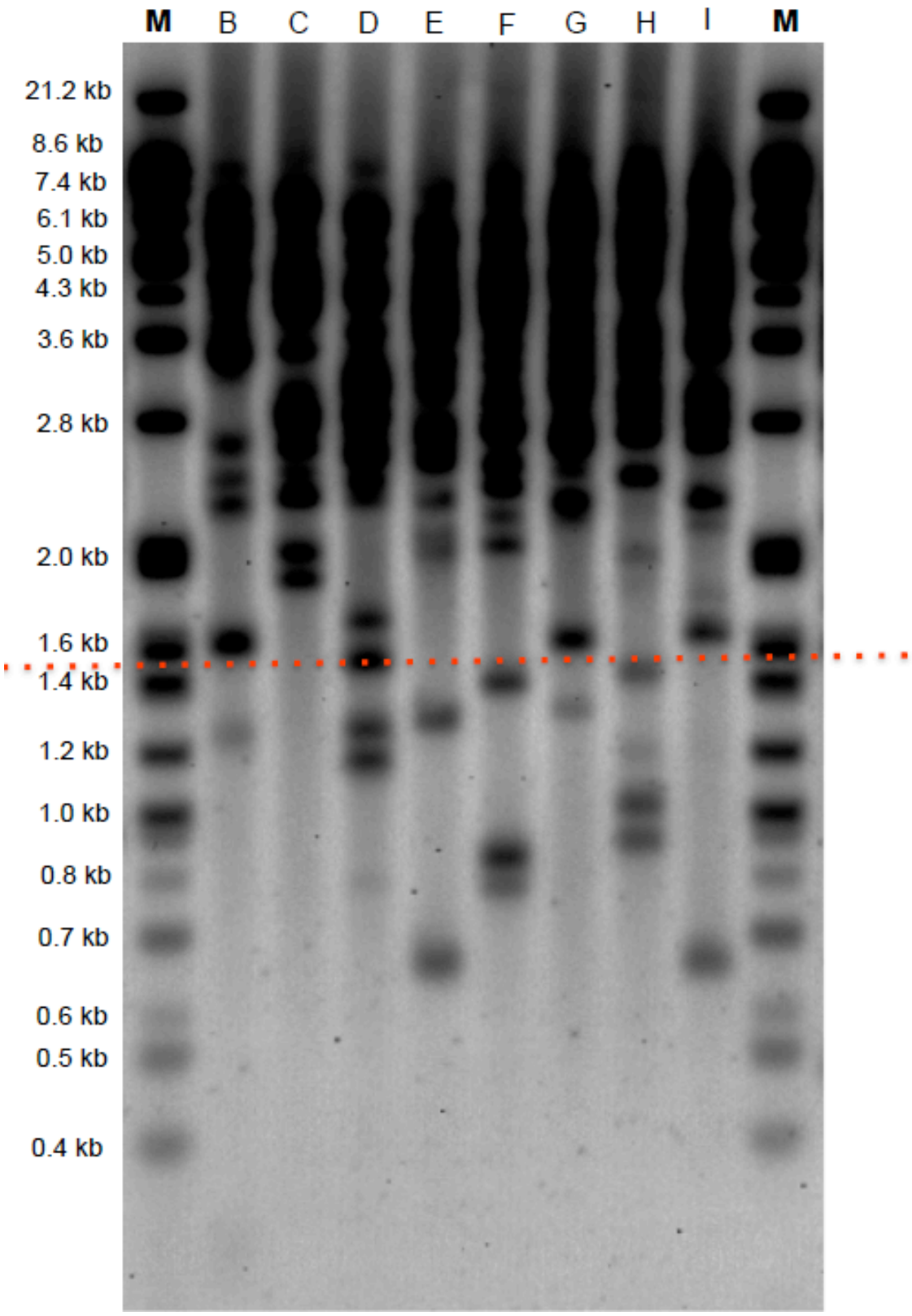

Figure 28 (b): U-STELA Gel 5_2 with markers

GEL 5_3

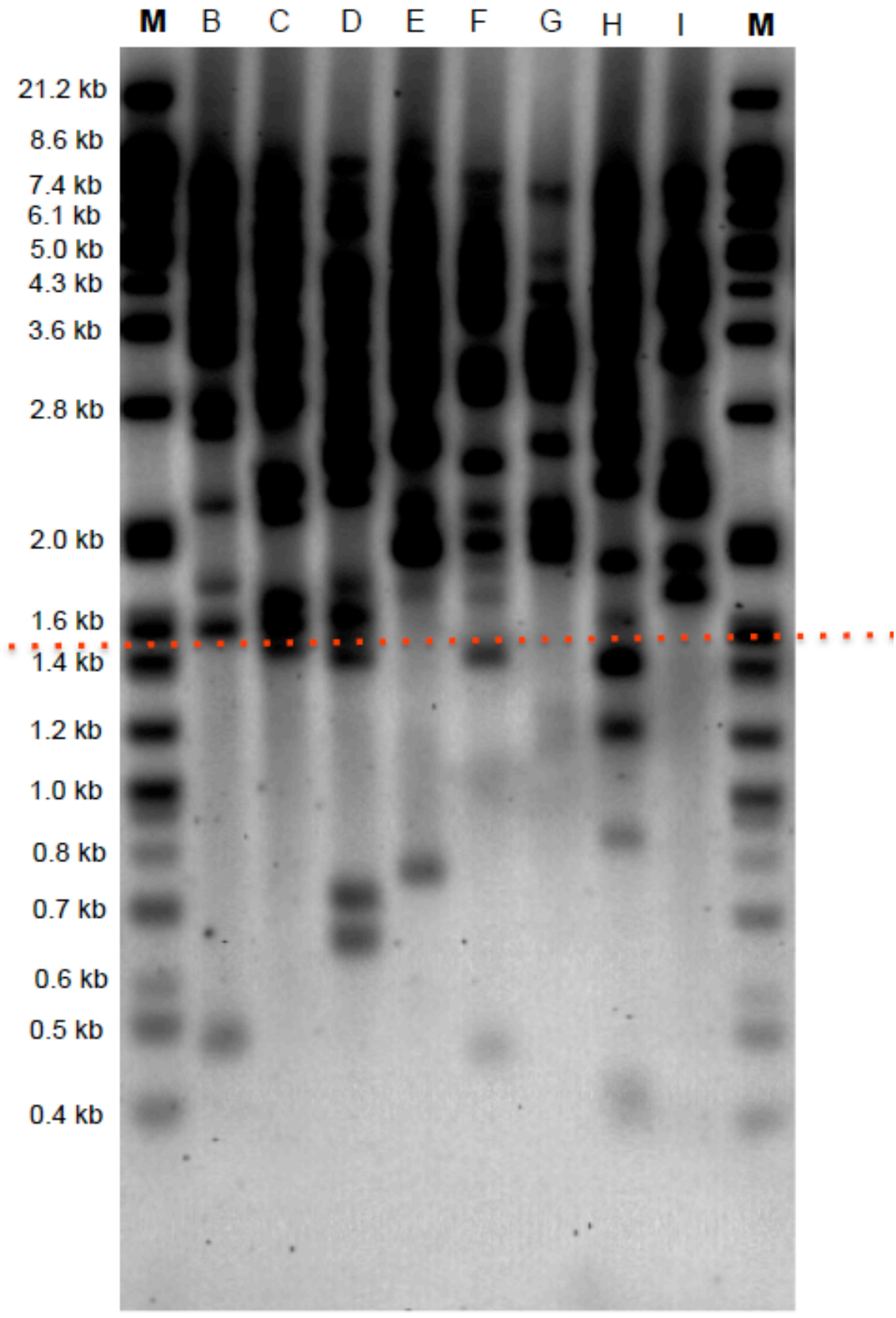

Figure 28 (c): U-STELA Gel 5_3 with markers

GEL 5_4

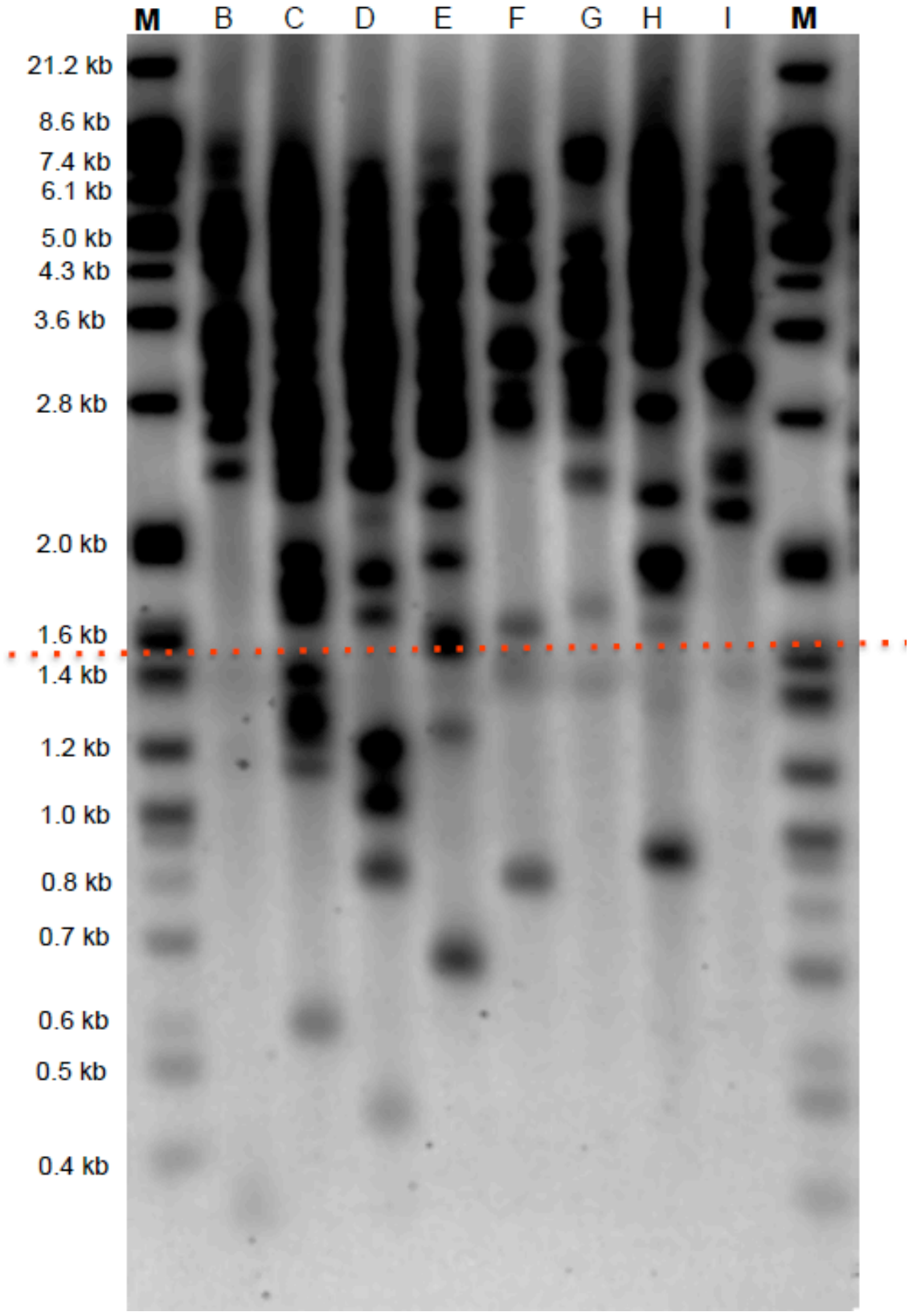

Figure 28 (d): U-STELA Gel 5_4 with markers

GEL 5_5

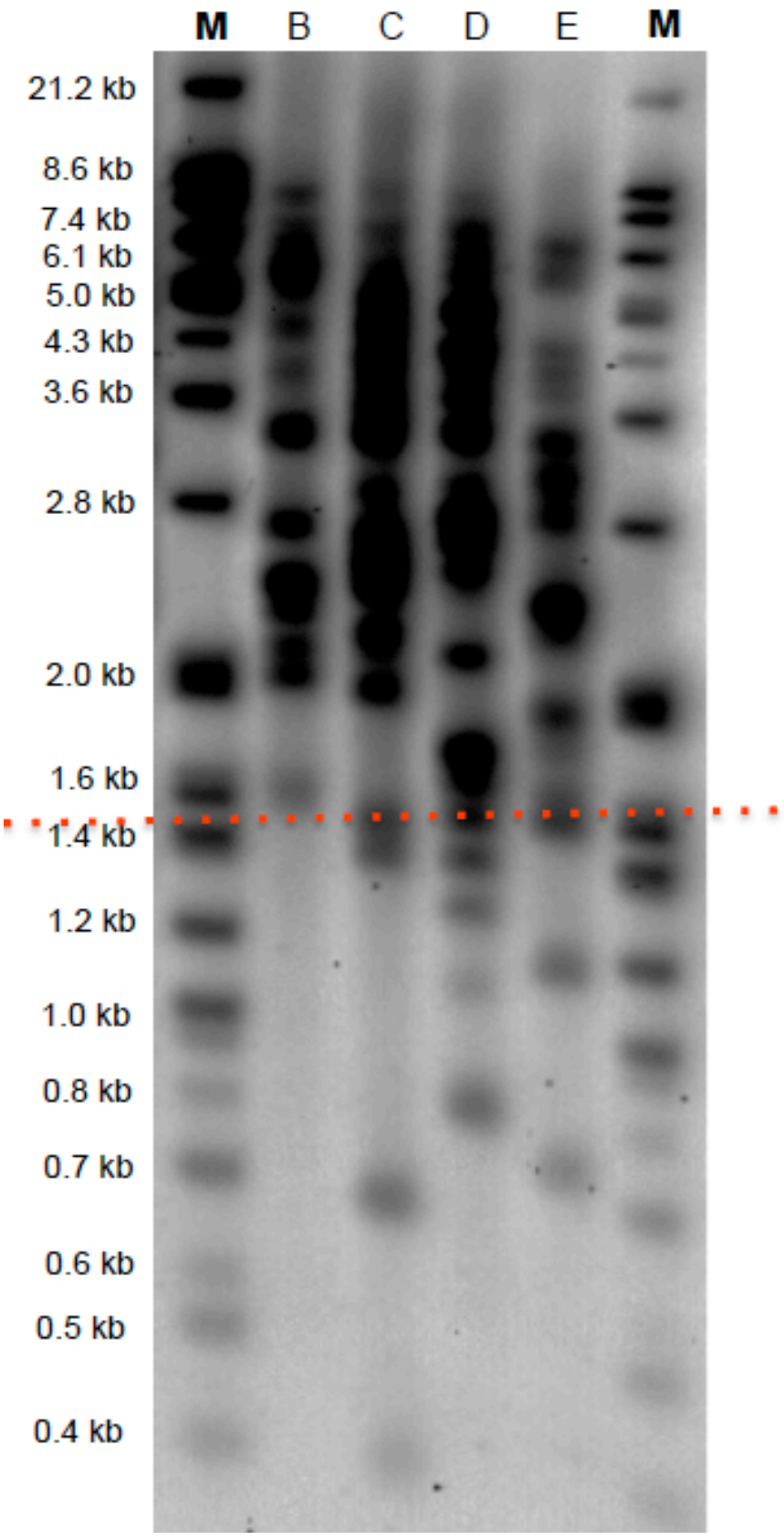

Figure 28 (e): U-STELA Gel 5_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 5_1	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1939.386	1706.422	2070.764	1979.588	2035.075	2094.904	2107.079	992	21226
2	8576	1004.277	1649.489	1889.285	1716.100	1288.012	1706.422	1900		8576
3	7427		1350.859	1677.714	1612.593	299.126	1342.839	1785.401		7427
4	6106		908.718	1411.004	1257.693		836.735	1042.025		6106
5	5007		748.237		1149.771			963.122		5007
6	4268				1061.428			583.421		4268
7	3585				641.431					3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	947									947
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 29 (a): Telomere bands corresponding to Gel 5_1

Band Gel 5_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1559.309	1949.359	1655.537	2013.489	1989.768	1578.097	1939.386	1757.702	21226
2	8576	1224.814	1843.955	1486.381	1959.383	1416.865	1313.655	1811.125	1606.703	8576
3	7427			1248.430	1280.628	892.921		1451.207	668.026	7427
4	6106			1149.205	659.041	836.447		1178.914		6106
5	5007			831				1017.706		5007
6	4268							934.706		4268
7	3585									3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	947									947
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 29 (b): Telomere bands corresponding to Gel 5_2

Band Gel 5_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1747.208	1665.480	1736.777	2133.545	2119.798	2106.141	1843.955	1843.955	21226
2	8576	1559.309	1578.097	1616.353	1888.656	1943.812	1977.332	1597.110	1705.855	8576
3	7427	467.487	1473.092	1527.625	1747.208	1832.946	1888.656	1420.505	1364.801	7427
4	6106			1420.505	798.942	1695.670	1516.559	1171.433		6106
5	5007			738.489		1441.311	1164	875.589		5007
6	4268			652.984		1042.025	972.458	374.619		4268
7	3585					455.693				3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	947									947
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 29 (c): Telomere bands corresponding to Gel 5_3

Band Gel 5_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1889.285	1878.630	1805.711	1857.501	1603.499	1677.714	1857.501	1391.116	21226
2	8576	1400	1755.364	1640.187	1558.791	1430.870	1382.289	1785.401		8576
3	7427	305.358	1649.489	1382.289	1373.517	849.296		1603.499		7427
4	6106		1400	1171.433	1224.814			1313.655		6106
5	5007		1256.403	1033.987	682.526			906.637		5007
6	4268		1116.734	855.484						4268
7	3585		579.036	422.065						3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1550									1550
12	1400									1400
13	1164									1164
14	992									992
15	947									947
16	831									831
17	710									710
18	564									564
19	492									492
20	359									359

Table 29 (d): Telomere bands corresponding to Gel 5_4

Band Gel 5_5	A(M)	B	C	D	E	J(M)
1	21226	1966.095	1900	2048.597	1805.711	21226
2	8576	1567.632	1462.421	1696.799	1716.100	8576
3	7427		1382.875	1594.456	1527.625	7427
4	6106		663.518	1473.092	1462.421	6106
5	5007		344.784	1340.975	1071.387	5007
6	4268			1215.236	705.209	4268
7	3585			1040.128		3585
8	2799			805.253		2799
9	2000					2000
10	1900					1900
11	1550					1550
12	1400					1400
13	1164					1164
14	992					992
15	947					947
16	831					831
17	710					710
18	564					564
19	492					492
20	359					359

Table 29 (e): Telomere bands corresponding to Gel 5_5

GEL 6

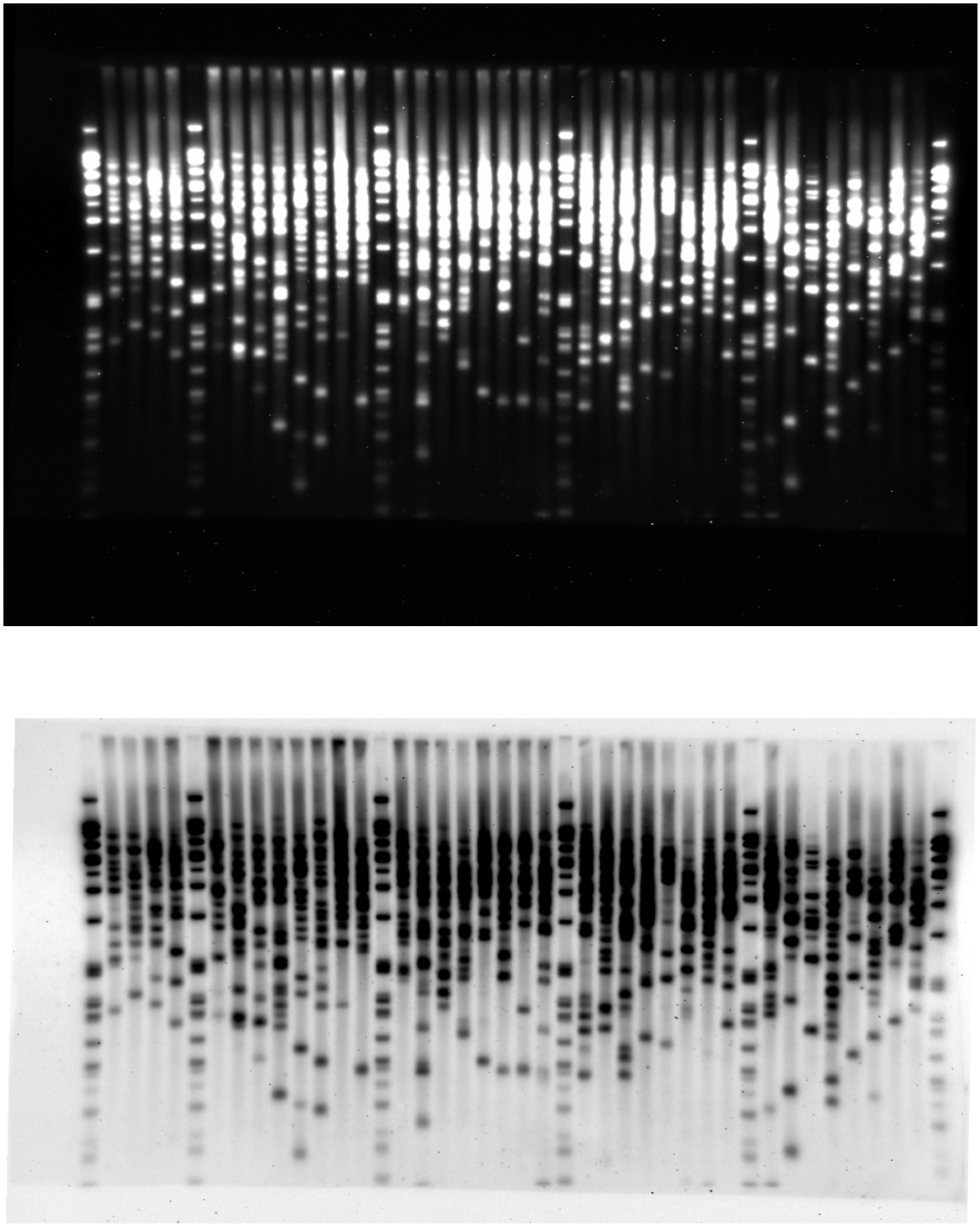

Figure 29: U-STELA Gel 6

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 6_1

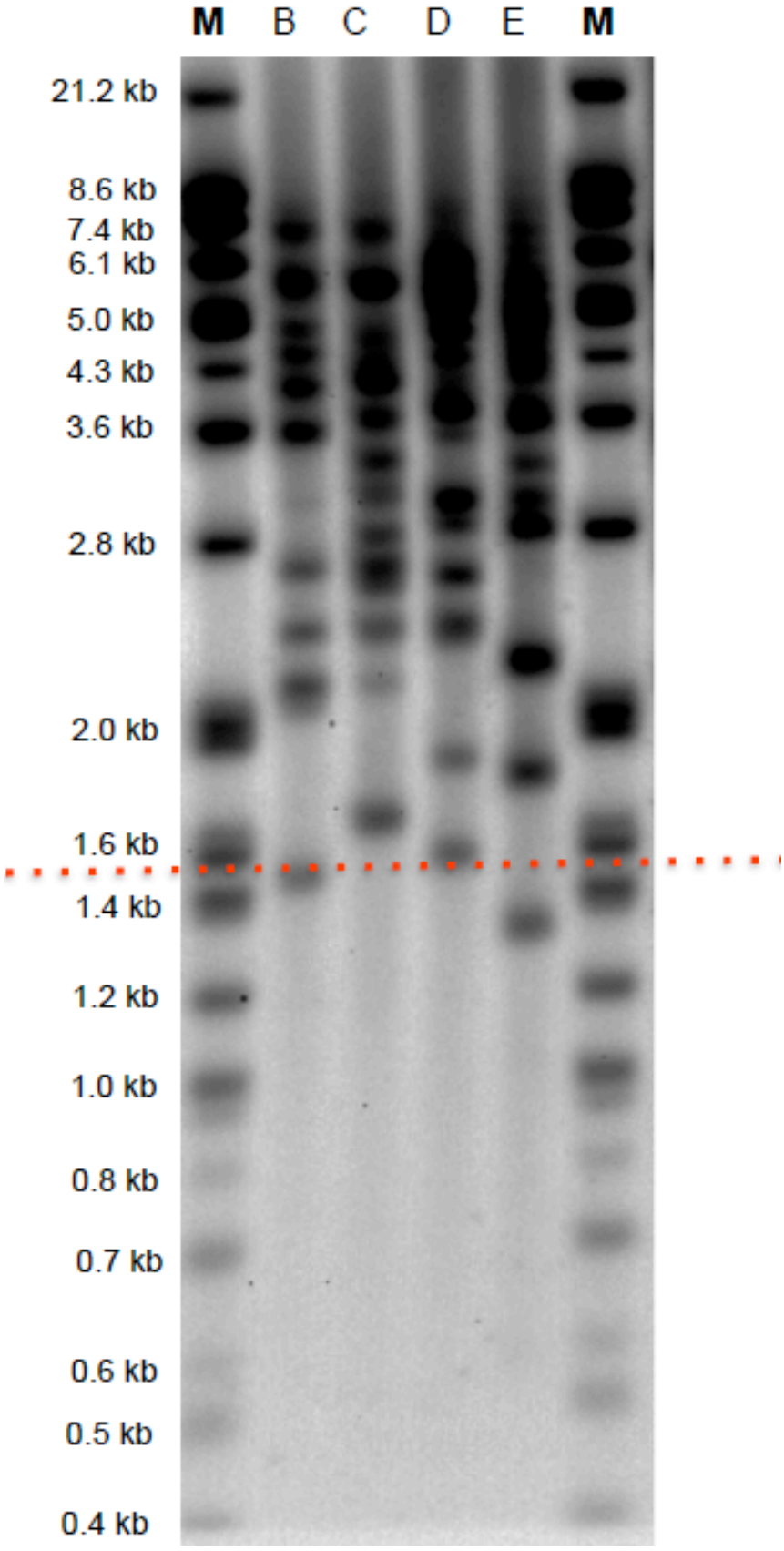

Figure 29 (a): U-STELA Gel 6_1 with markers

GEL 6_2

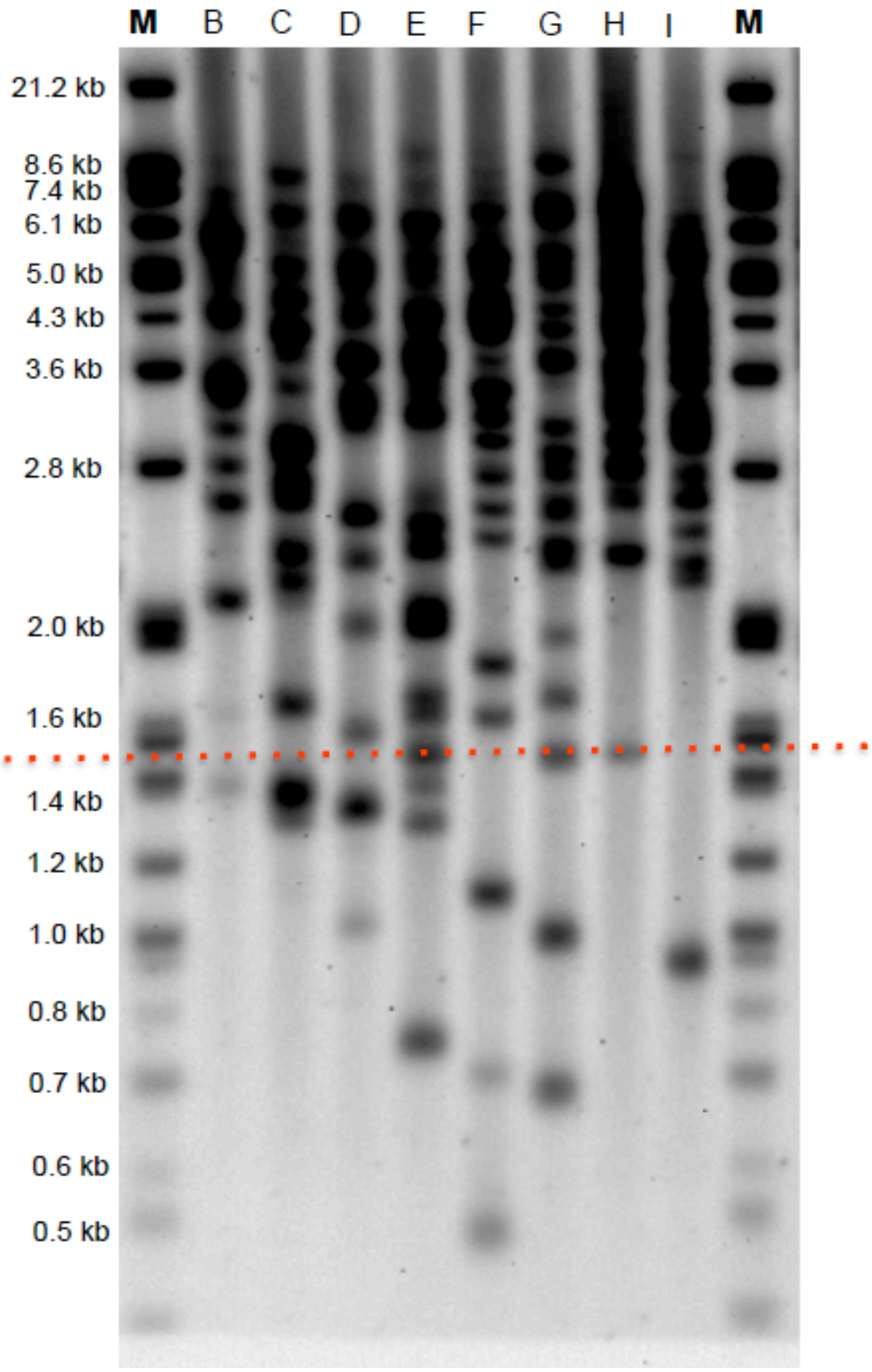

Figure 29 (b): U-STELA Gel 6_2 with markers

GEL 6_3

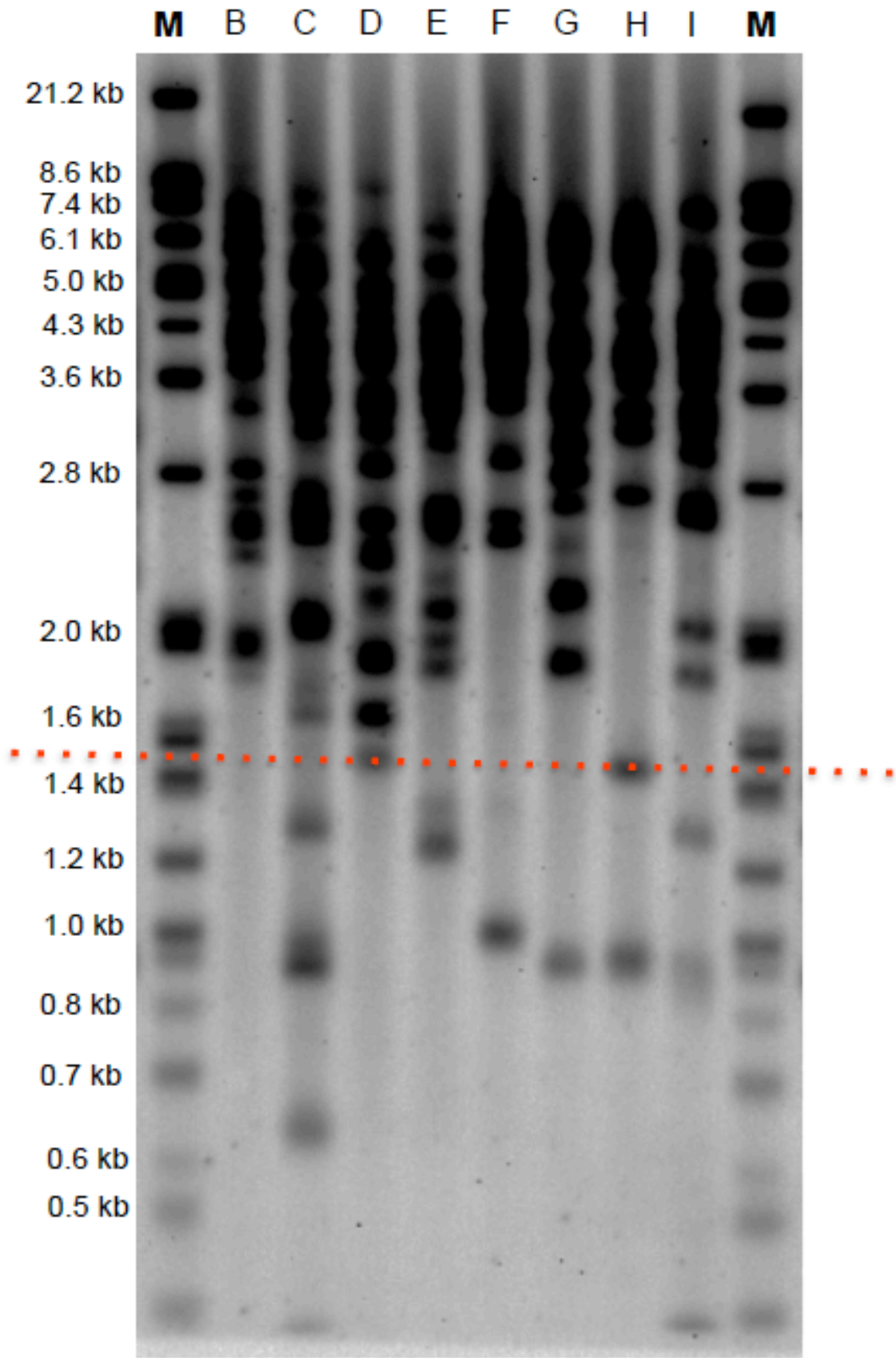

Figure 29 (c): U-STELA Gel 6_3 with markers

GEL 6_4

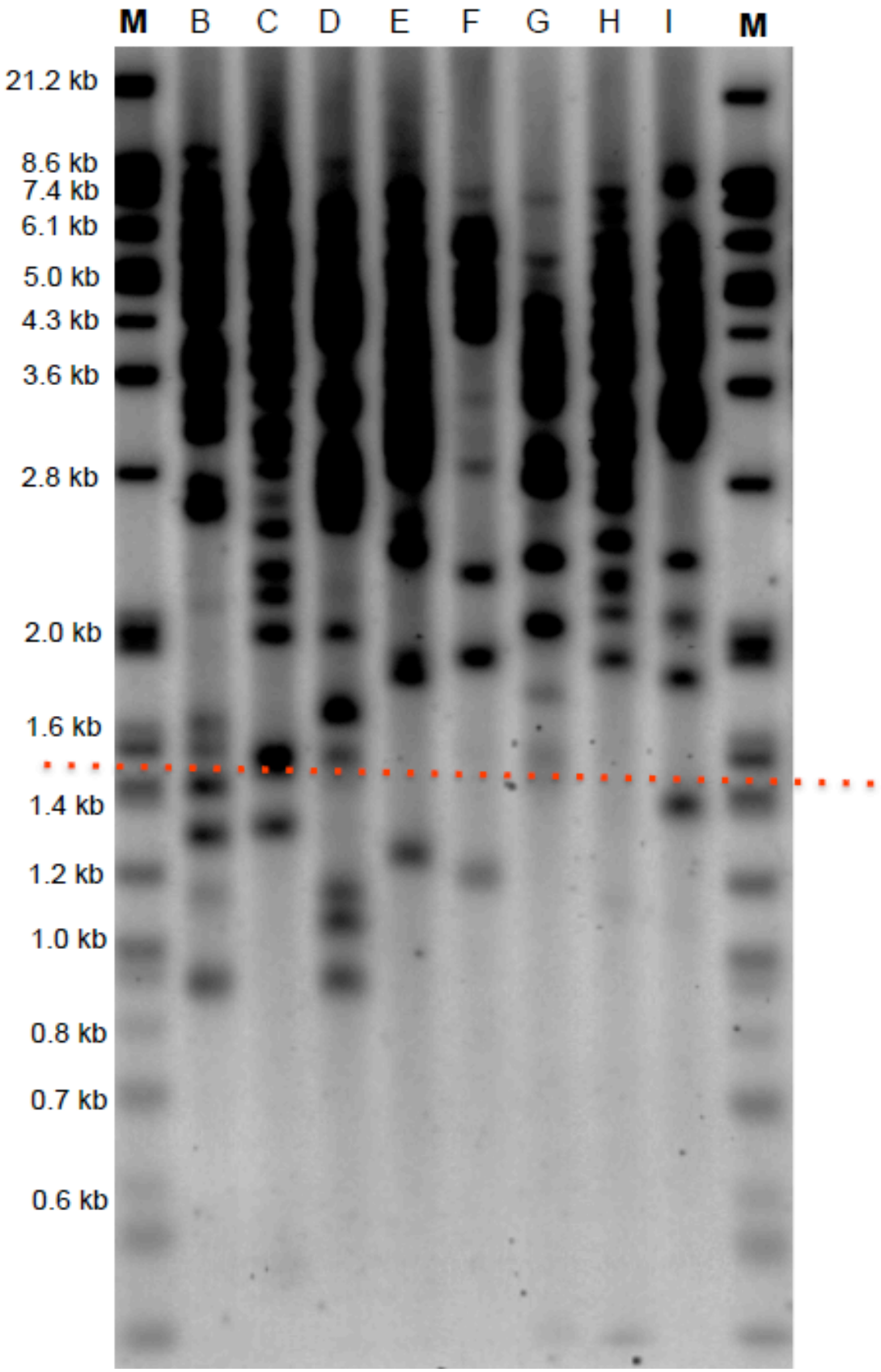

Figure 29 (d): U-STELA Gel 6_4 with markers

GEL 6_5

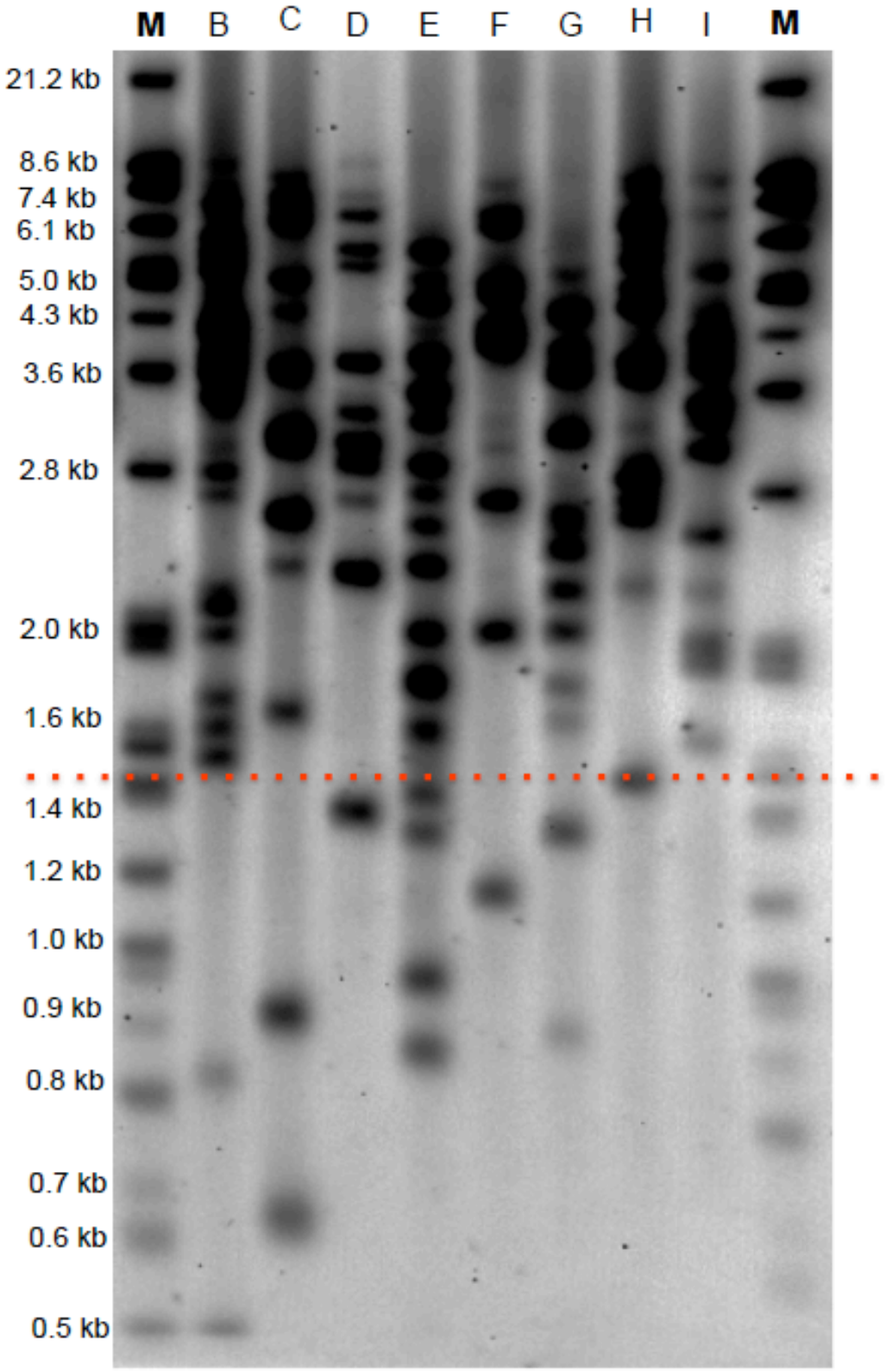

Figure 29 (e): U-STELA Gel 6_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 6_1	A(M)	B	C	D	E	F(M)
1	21226	2080.670	1614.418	1791.469	1759.339	21226
2	8576	1436.081		1520.699	1305.544	8576
3	7427				560.162	7427
4	6106					6106
5	5007					5007
6	4268					4268
7	3585					3585
8	2799					2799
9	2000					2000
10	1900					1900
11	1550					1550
12	1400					1400
13	1164					1164
14	992					992
15	947					947
16	831					831
17	710					710
18	564					564
19	492					492
20	359					359

Table 30 (a): Telomere bands corresponding to Gel 6_1

Band Gel 6_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1678.383	1692.318	2009.626	2019.299	1823.054	1942.229	1566.461	1007.989	21226
2	8576	1508.026	1498.778	1617.082	1706.368	1657.696	1713.437			8576
3	7427		1475.904	1487.297	1671.459	1323.206	1557.764			7427
4	6106		1401.784	1217.416	1557.764	841.929	1179.018			6106
5	5007				1510.347	557.041	821.249			5007
6	4268				1471.372					4268
7	3585				898.774					3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 30 (b): Telomere bands corresponding to Gel 6_2

Band Gel 6_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1891.789	1985.398	1835.296	2080.670	1646.961	1835.297	1527.705	1985.399	21226
2	8576	1803.776	1742.349	1661.289	1900	1482.729	985.739	992	1788.219	8576
3	7427		1661.289	1549.116	1811.605	1164			1457.857	7427
4	6106		1549.116		1480.451				979.519	6106
5	5007		1462.348		1446.689				54.184	5007
6	4268		1040.744							4268
7	3585		983.662							3585
8	2799		754.299							2799
9	2000		54.184							2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 30 (c): Telomere bands corresponding to Gel 6_3

Band Gel 6_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1861.129	2000	2020.168	1838.189	1853.451	2020.168	1845.804	2061.115	21226
2	8576	1650.857	1567.487	1685.336	1793.155	1571.599	1742.006	1323.206	1785.757	8576
3	7427	1584	1478.555	1571.599	1450.031	1429	1567.487	359	1497.882	7427
4	6106	1518.974	1419.869	1473.762			492			6106
5	5007	1468.985	537.759	1357.571						5007
6	4268	1348.897		1257.065						4268
7	3585	986.011		988.003						3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 30 (d): Telomere bands corresponding to Gel 6_4

Band Gel 6_5	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1974.517	1685.336	1488.905	1949.359	2000	2000	1523.457	1884.355	21226
2	8576	1720.535	957.610	1293.985	1763.746	1355.326	1756.469		1830.606	8576
3	7427	1637.263	622.483		1630.509		1657.696		1597.151	7427
4	6106	1566.461			1505.459		1470.210			6106
5	5007	858.595			1470.210		933.834			5007
6	4268	492			1319.927					4268
7	3585				982.832					3585
8	2799				903.821					2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 30 (e): Telomere bands corresponding to Gel 6_5

GEL 7

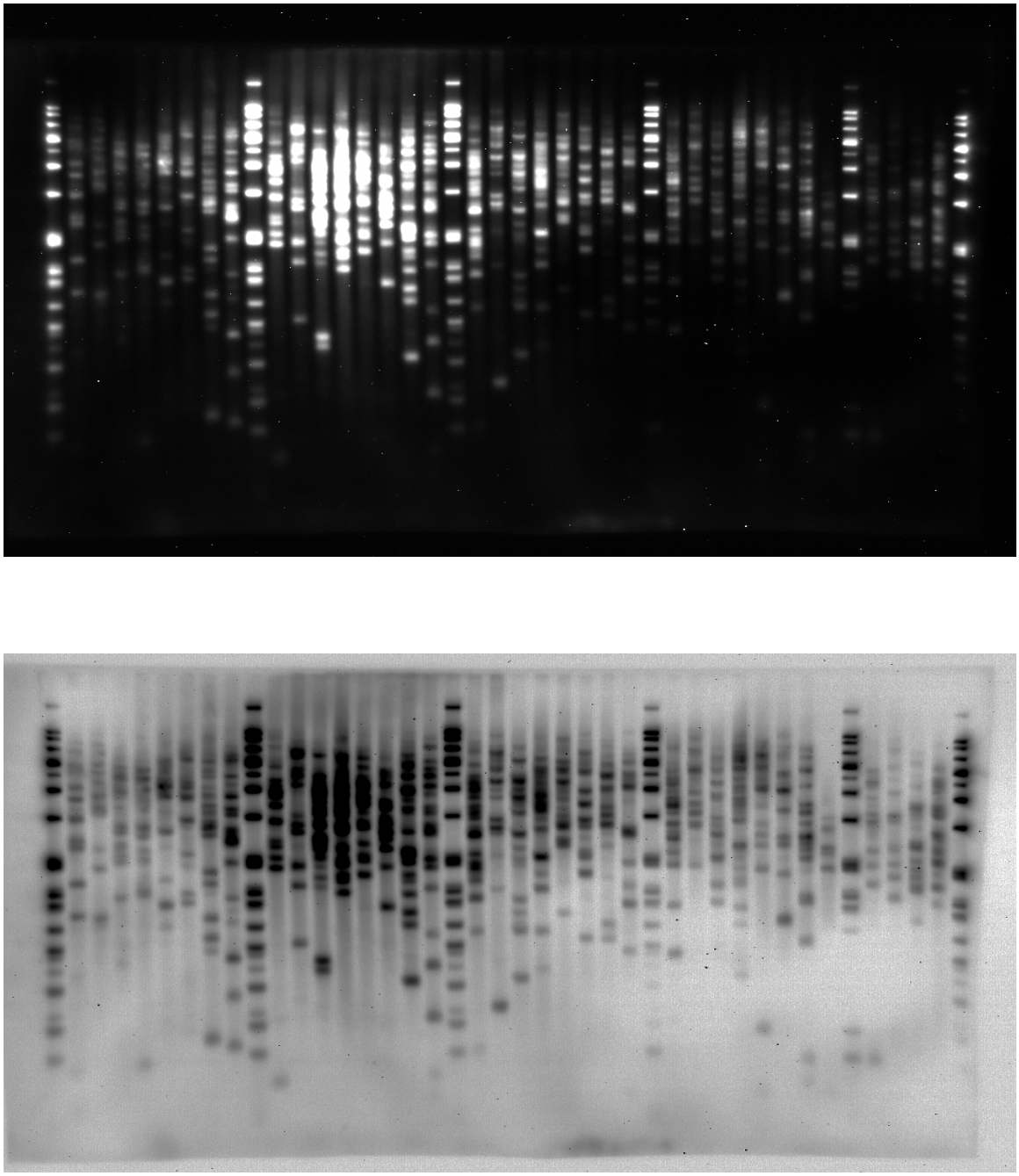

Figure 30: U-STELA Gel 7

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 7_1

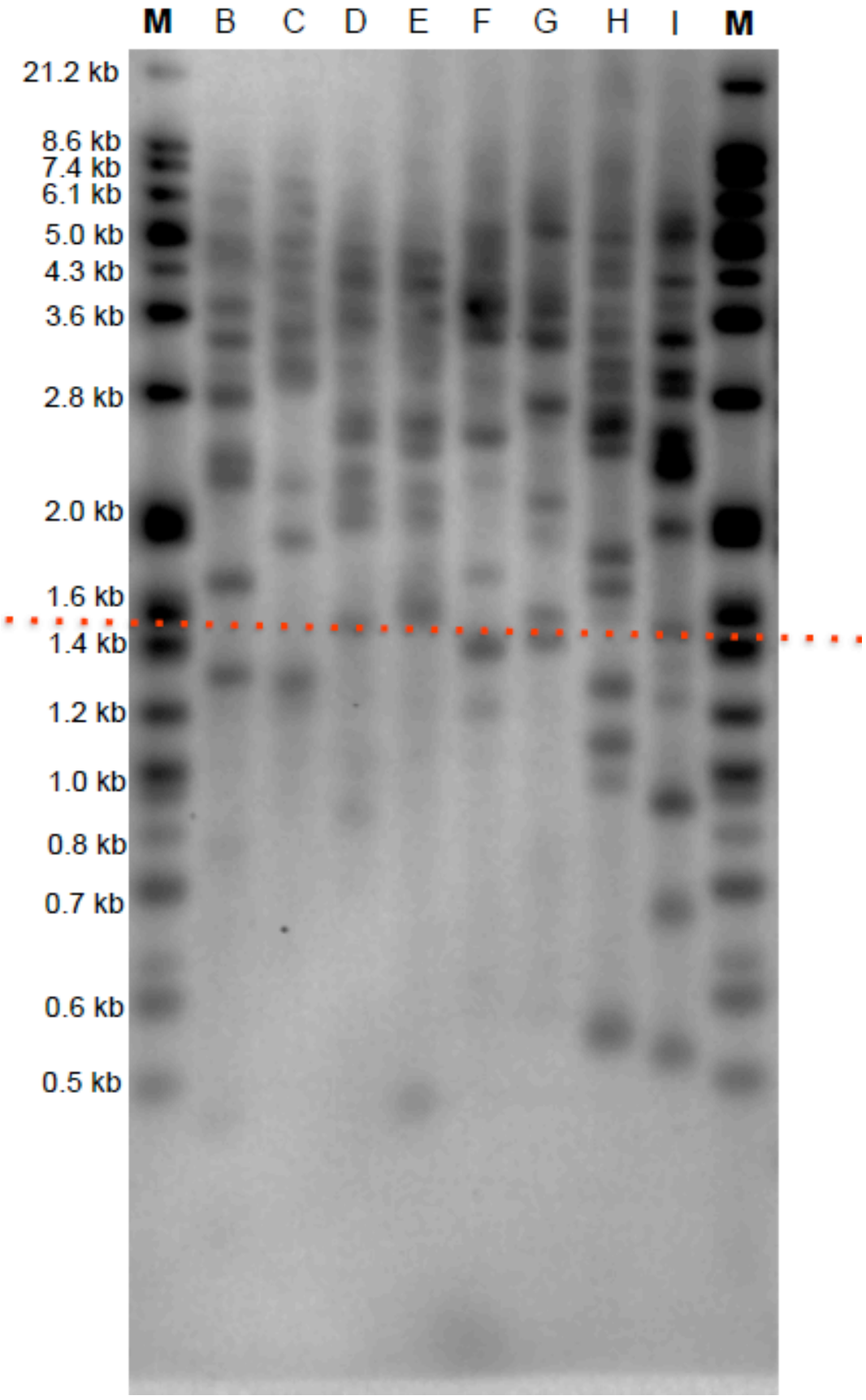

Figure 30 (a): U-STELA Gel 7_1 with markers

GEL 7_2

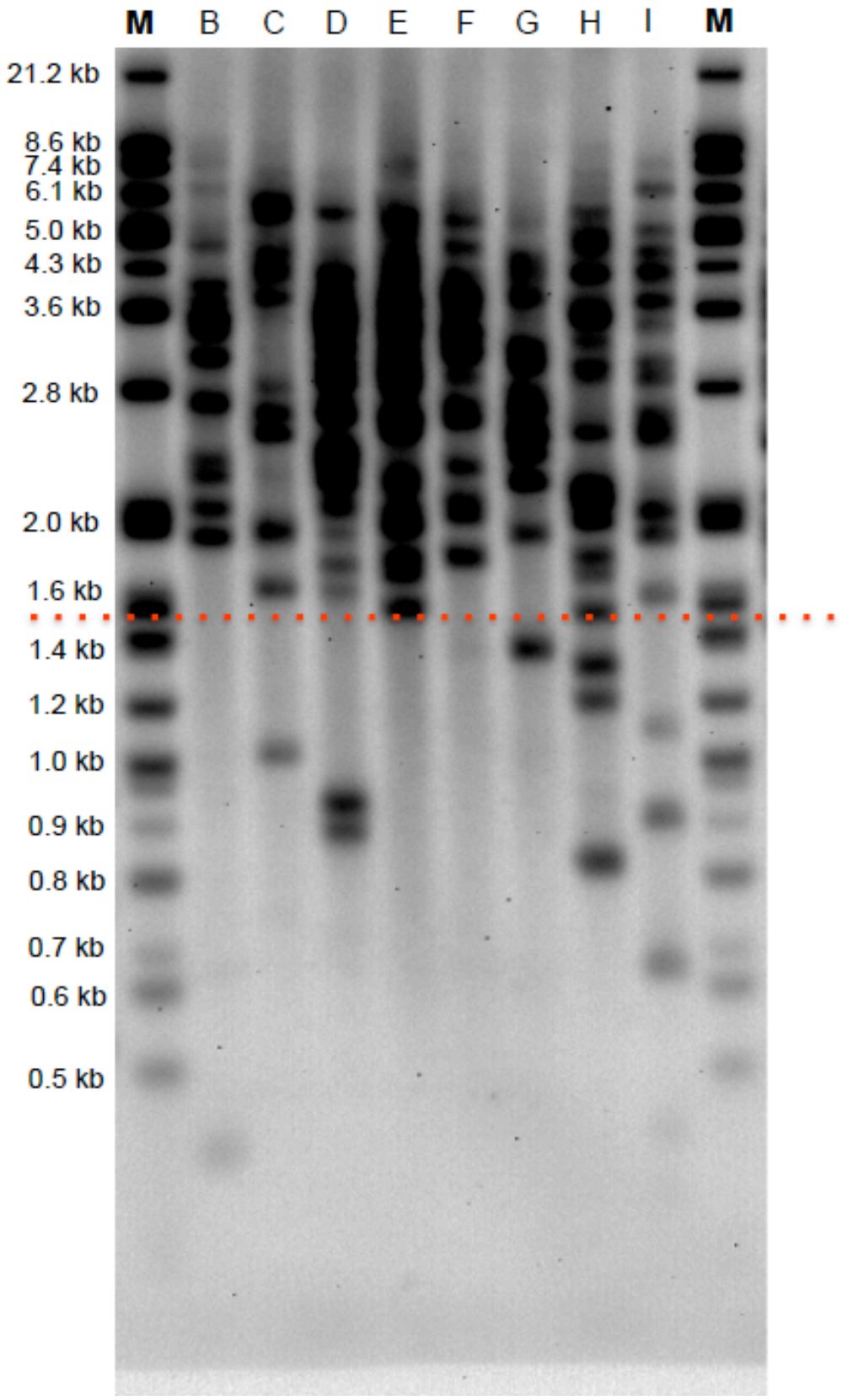

Figure 30 (b): U-STELA Gel 7_2 with markers

GEL 7_3

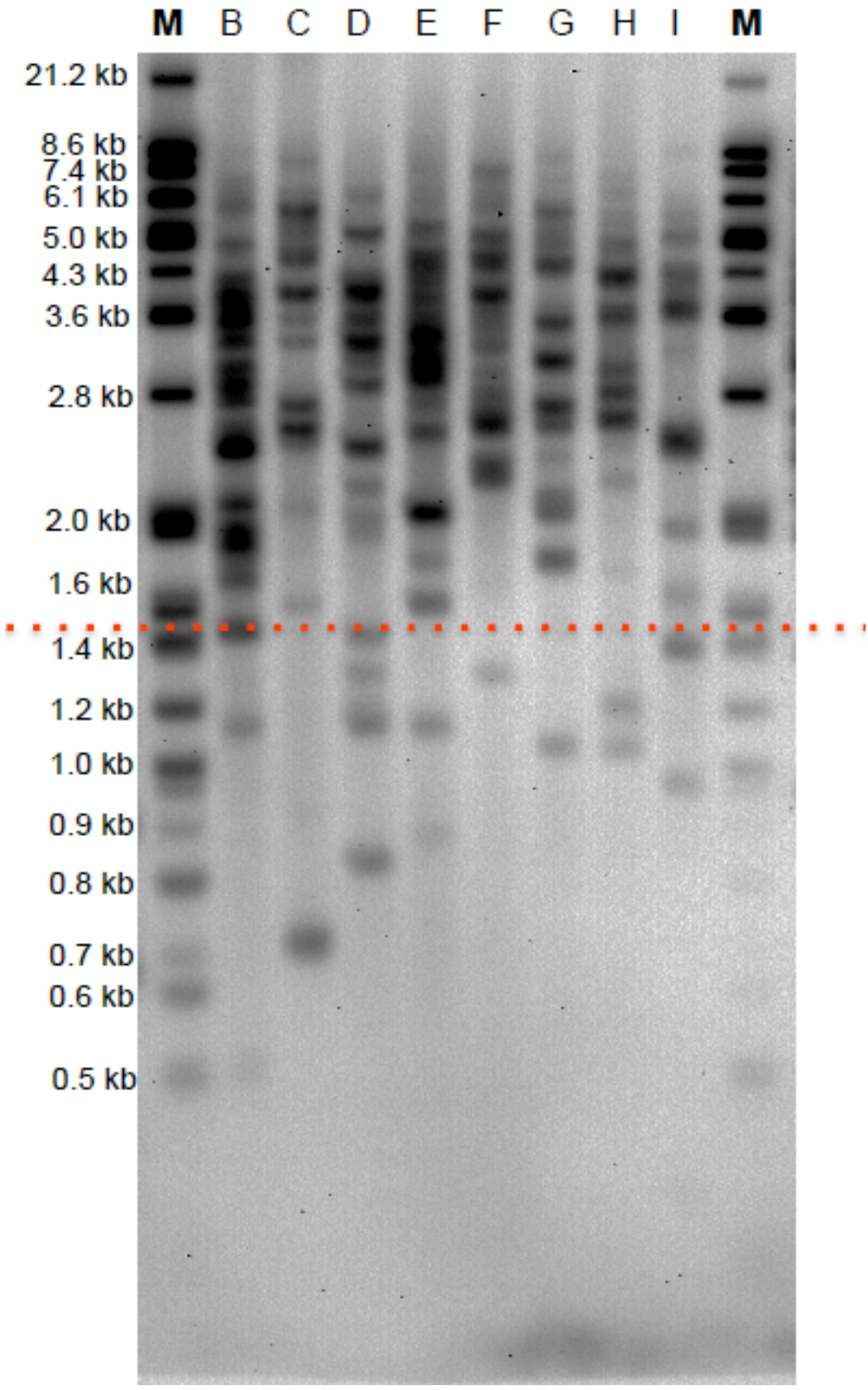

Figure 30 (c): U-STELA Gel 7_3 with markers

GEL 7_4

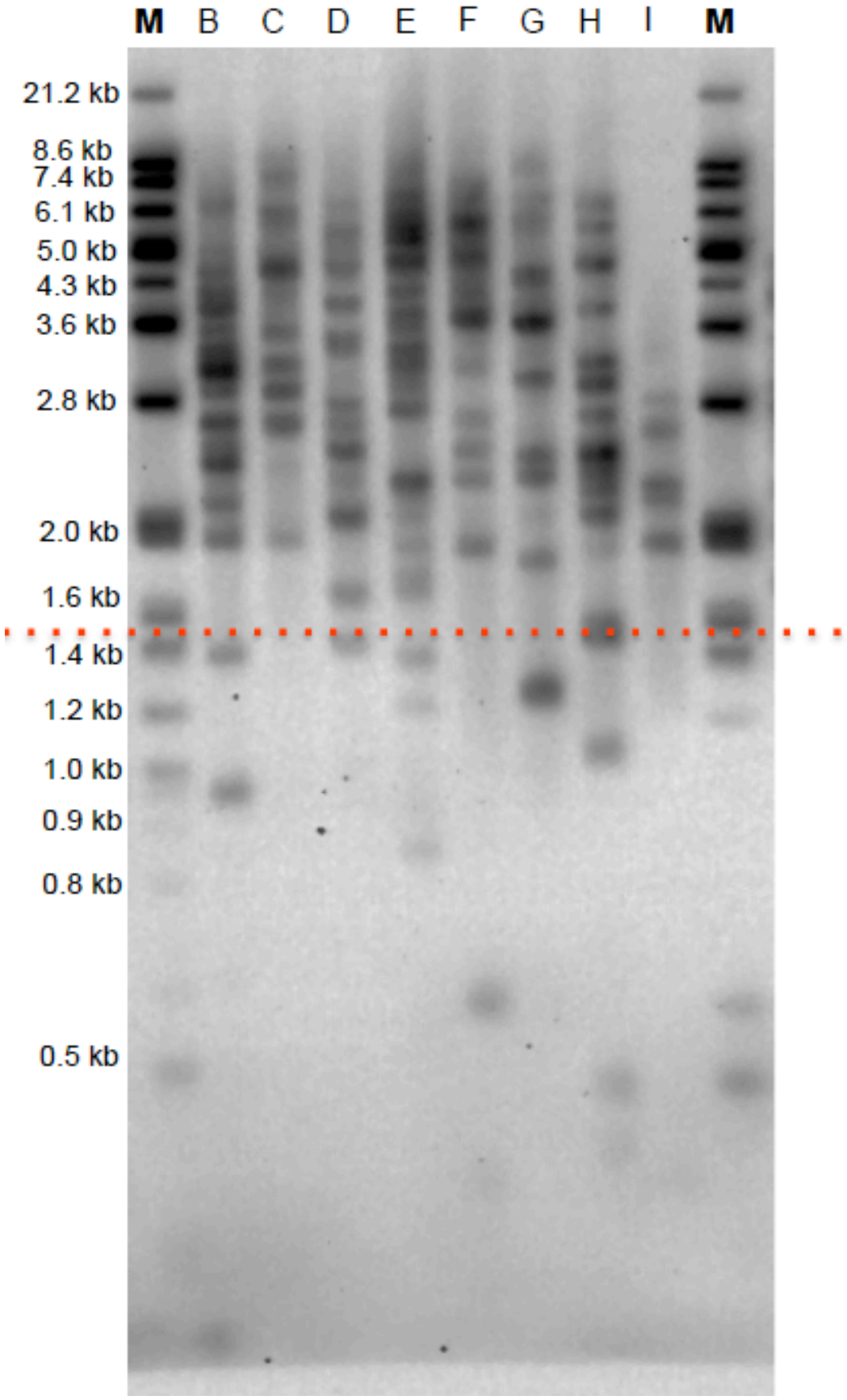

Figure 30 (d): U-STELA Gel 7_4 with markers

GEL 7_5

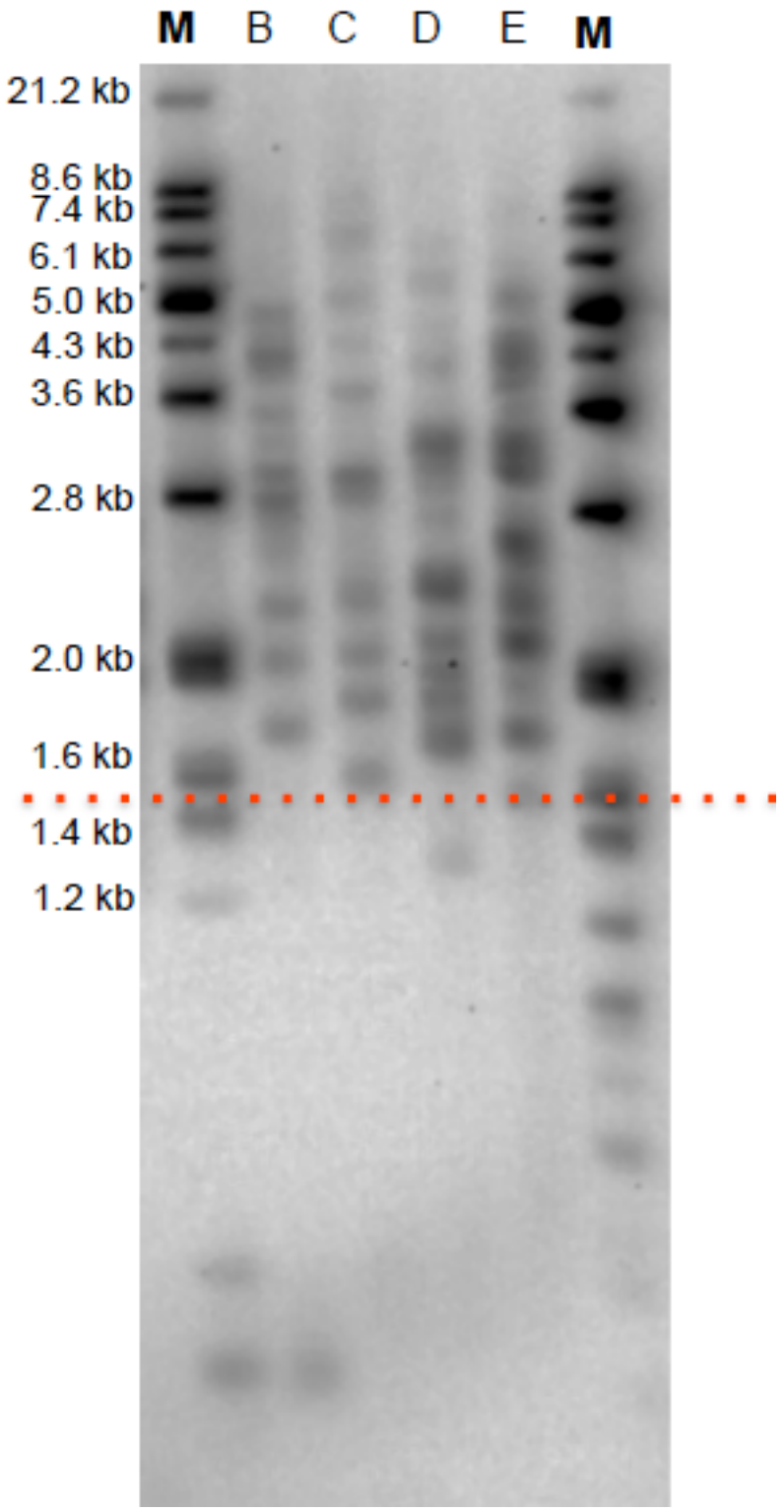

Figure 30 (e): U-STELA Gel 7_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 7_1	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1696.862	1872.183	1966.095	1988.634	1730.561	2060.930	1808.851	1932.765	21226
2	8576	1475.538	1467.217	1556.031	1756.273	1512.147	1890.682	1680.259	1551.417	8576
3	7427	918.869	1442.533	1225.245	1599.651	1434.398	1579.304	1584	1489.512	7427
4	6106			972.798	39.537		1524.023	1461.695	1447.982	6106
5	5007						887.106	1280.298	984.755	5007
6	4268							1123.367	797.095	4268
7	3585							536.323	519.252	3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 31 (a): Telomere bands corresponding to Gel 7_1

Band Gel 7_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2000	1900	2037.697	1939.386	1969.459	1890.424	1959.383	1989.768	21226
2	8576	1871.416	1666.093	1900	1752.440	1797.278	1505.869	1815.532	1900	8576
3	7427	0.0042	1216.302	1770.239	1584	1505.869		1734.819	1657.696	7427
4	6106			1657.696				1579.304	1308.746	6106
5	5007			971.491				1484.775	962.513	5007
6	4268			949.201				1437.665	663.518	4268
7	3585							980.553	0.0528	3585
8	2799							882.289		2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 31 (b): Telomere bands corresponding to Gel 7_2

Band Gel 7_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1942.229	1600.088	1985.398	2023.727	1700.109	2047.735	1743.608	1942.229	21226
2	8576	1852.599	970.424	1524.669	1788.219	1472.759	1779.206	1434.398	1649.341	8576
3	7427	1717.377	736.782	1467.217	1616.340		1255.872	1246.367	1506.456	7427
4	6106	1544.196		1354.995	1354.995				1046.306	6106
5	5007	1344.740		878.234	928.153					5007
6	4268	497.334								4268
7	3585									3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 31 (c): Telomere bands corresponding to Gel 7_3

Band Gel 7_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1890.682	1853.865	2069.544	1863.002	1863.002	1808.851	1890.682	1881.409	21226
2	8576	1509.109		1672.019	1696.862	553.955	1457.110	1556.031		8576
3	7427	992		1515	1503.241			1261.678		7427
4	6106				1437.376			139.471		6106
5	5007				903.821			0.00581		5007
6	4268									4268
7	3585									3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 31 (d): Telomere bands corresponding to Gel 7_4

Band Gel 7_5	A(M)	B	C	D	E	F(M)
1	21226	1970.903	1978.138	1942.229	1913.974	21226
2	8576	1768.357	1855.065	1872.909	1768.357	8576
3	7427		1645.835	1751.508	1591.601	7427
4	6106		509.535	1490.302		6106
5	5007					5007
6	4268					4268
7	3585					3585
8	2799					2799
9	2000					2000
10	1900					1900
11	1584					1584
12	1515					1515
13	1429					1429
14	1164					1164
15	992					992
16	947					947
17	831					831
18	710					710
19	564					564
20	492					492

Table 31 (d): Telomere bands corresponding to Gel 7_5

GEL 8

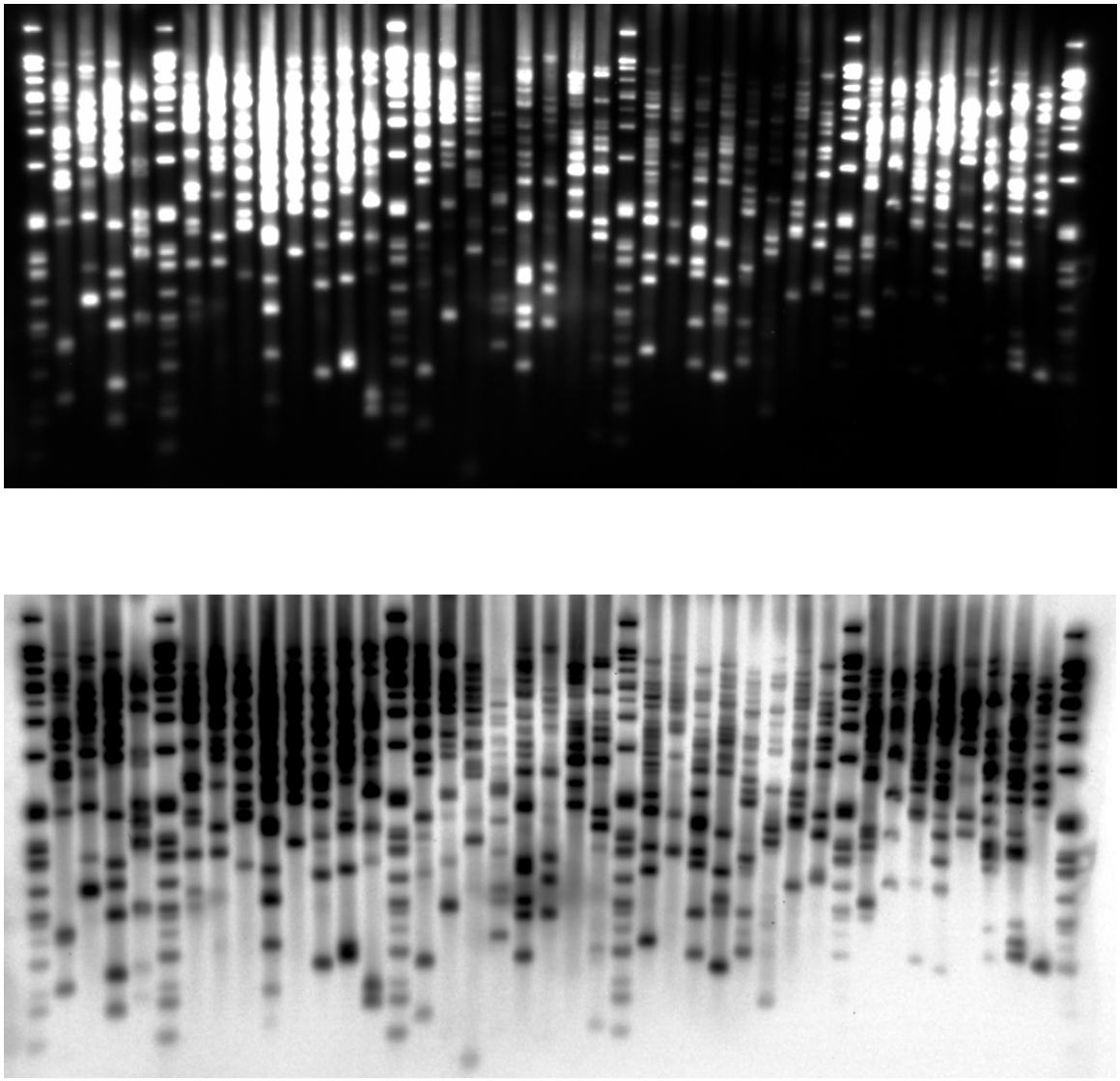

Figure 31: U-STELA Gel 8

For purposes of counting the bands, the gel was divided into sections, which are shown below.

GEL 8_1

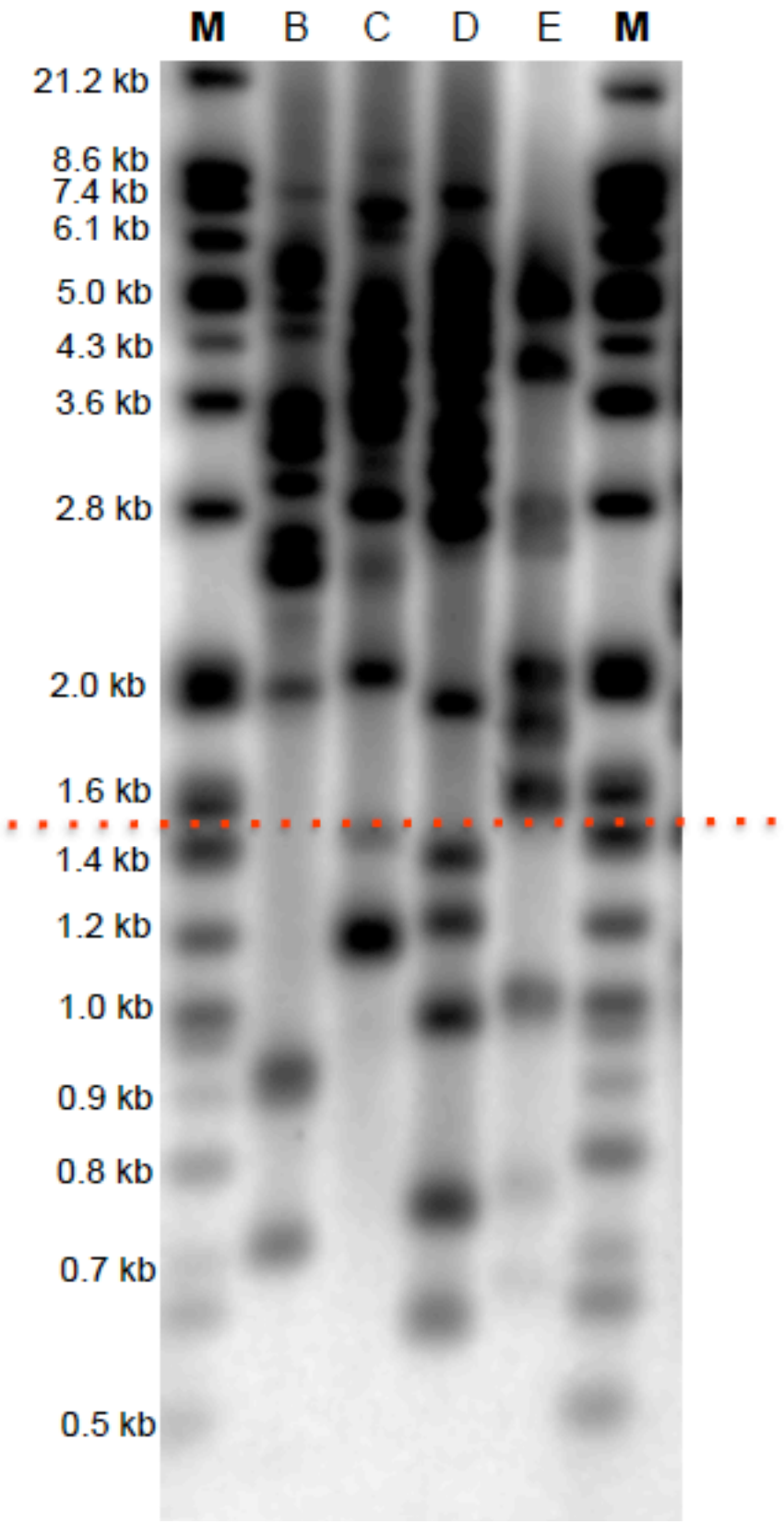

Figure 31 (a): U-STELA Gel 8_1 with markers

GEL 8_2

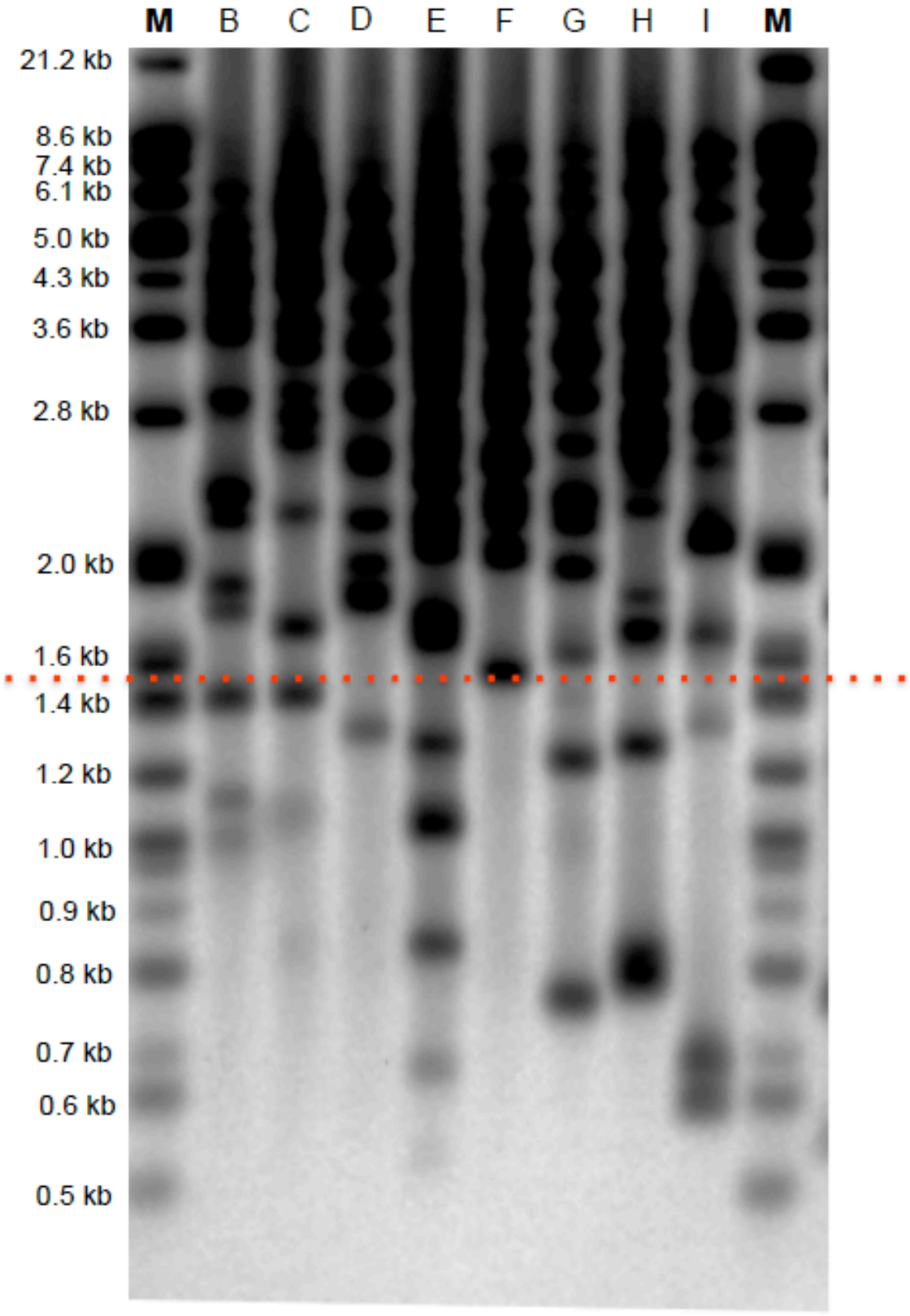

Figure 31 (b): U-STELA Gel 8_2 with markers

GEL 8_3

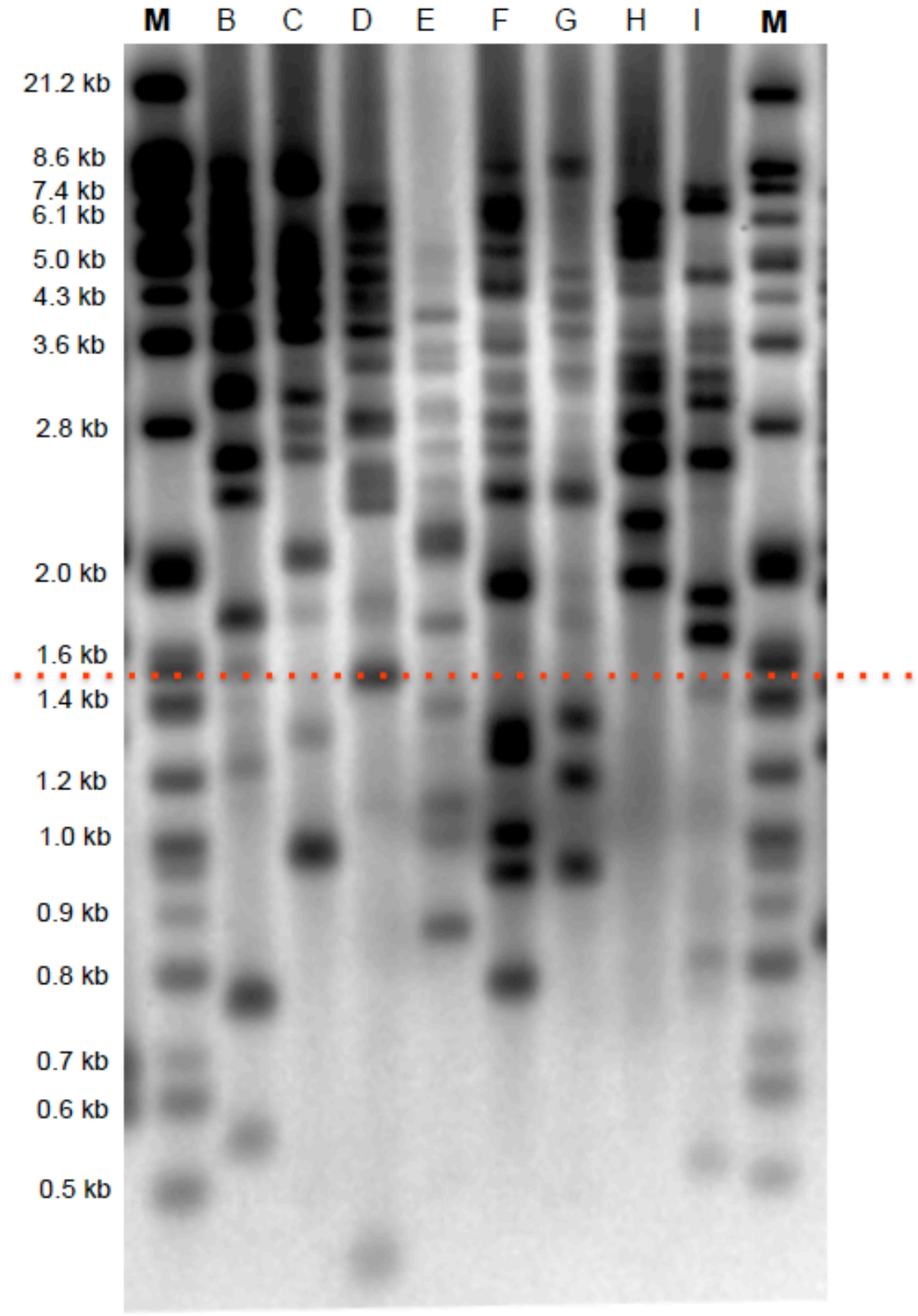

Figure 31 (c): U-STELA Gel 8_3 with markers

GEL 8_4

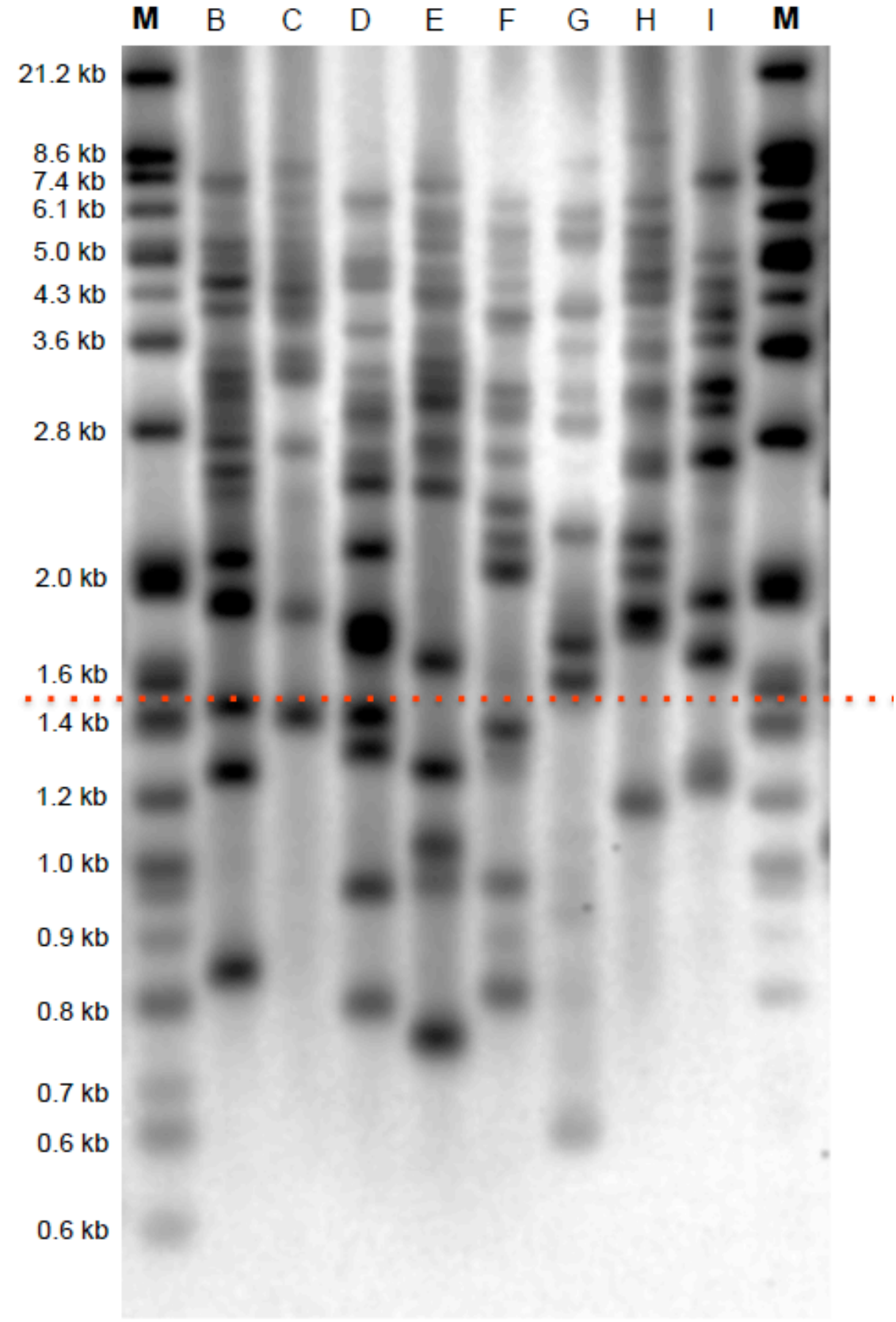

Figure 31 (d): U-STELA Gel 8_4 with markers

GEL 8_5

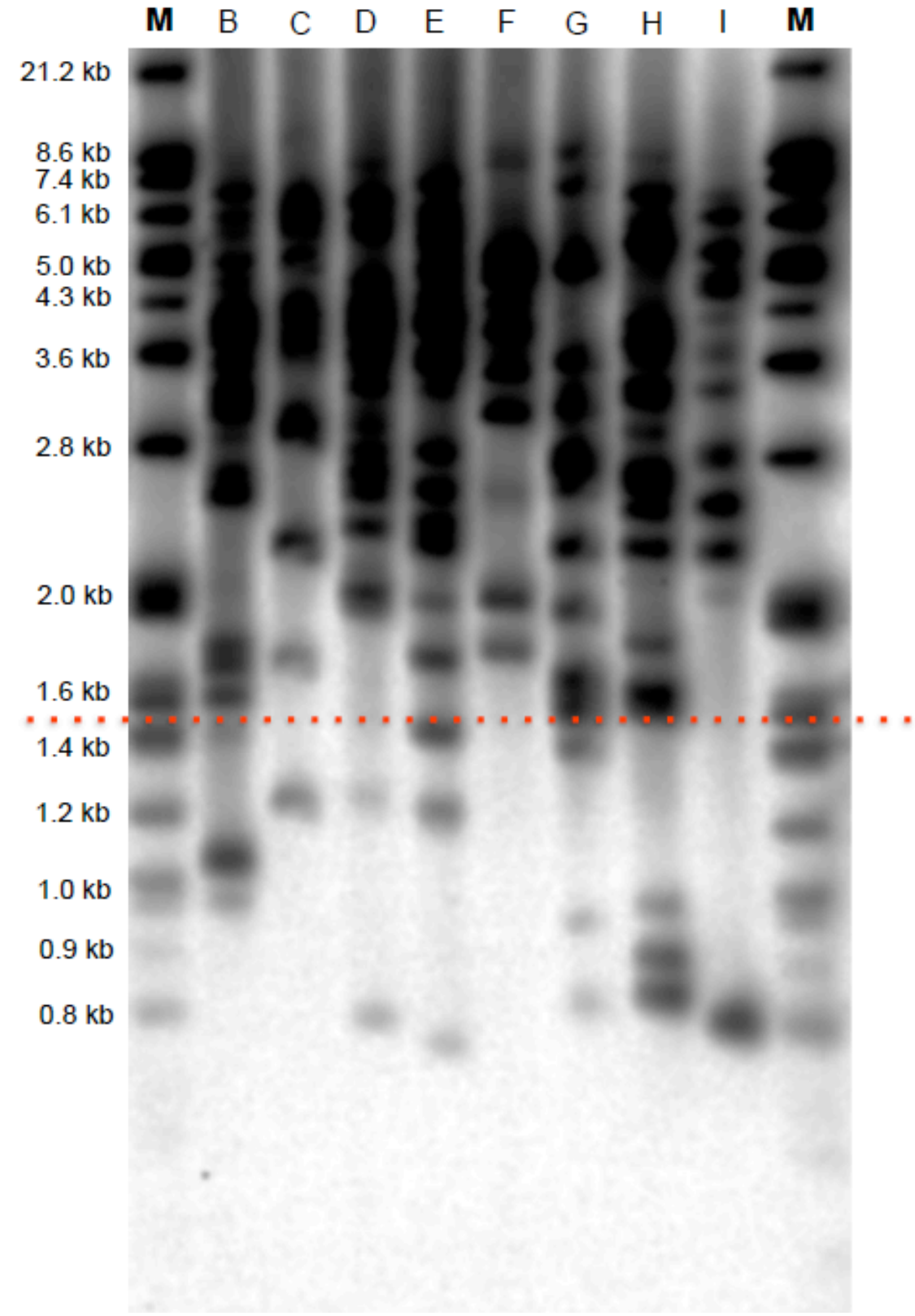

Figure 31 (e): U-STELA Gel 8_5 with markers

The corresponding bands counted using Vision Works LS Analysis software are shown below:

Band Gel 8_1	A(M)	B	C	D	E	F(M)
1	21226	1919.592	1979.588	1874.879	1979.588	21226
2	8576	963.336	1537.659	1506.972	1939.386	8576
3	7427	723.673	1429	1446.825	1817.547	7427
4	6106			1164	1626.732	6106
5	5007			777.333	1216.302	5007
6	4268			560.344	803.719	4268
7	3585				636.649	3585
8	2799					2799
9	2000					2000
10	1900					1900
11	1584					1584
12	1515					1515
13	1429					1429
14	1164					1164
15	992					992
16	947					947
17	831					831
18	710					710
19	564					564
20	492					492

Table 32 (a): Telomere bands corresponding to Gel 8_1

Band Gel 8_2	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1863.966	1710.085	1974.517	1768.357	1974.517	1936.900	1819.883	1734.819	21226
2	8576	1776.842	1524.023	1802.543	1669.641	1569.953	1622.369	1685.703	1685.703	8576
3	7427	1519.505	1276.101	1479.455	1463.332		1519.505	1460.663	1487.583	7427
4	6106	1322.036	860.755		1231.761		1444.745	831	683.274	6106
5	5007	1172.262			882.659		1164		578.613	5007
6	4268				666.019		794.465			4268
7	3585				524.011					3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 32 (b): Telomere bands corresponding to Gel 8_2

Band Gel 8_3	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1738.870	2011.828	1788.219	1722.725	1942.229	1873.599	1882.359	1813.417	21226
2	8576	1573.954	1755.167	1559.004	1504.306	1838.969	1738.870	1472.675	1683.015	8576
3	7427	1506.972	1467.468	1294.281	1294.281	1628.954	1491.045	1369.626	1524.669	7427
4	6106	1434.070	1053.300	0.00581	1180.583	1475.285	1408.928	1231.761	1294.281	6106
5	5007	780.304			905.119	1449.389	985.116		953.618	5007
6	4268	521.122				1303.467			847.875	4268
7	3585					1180.583			502.725	3585
8	2799					978.279				2799
9	2000					805.253				2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 32 (c): Telomere bands corresponding to Gel 8_3

Band Gel 8_4	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	1838.969	1813.417	1722.725	1651.907	1598.845	1771.616	1796.579	1856.204	21226
2	8576	1537.659	1519.505	1524.023	1462.280	1501.645	1698.788	1418.570	1667.388	8576
3	7427	1457.110		1483.144	1243.328	1470.069	1584		1451.958	7427
4	6106	1172.558		1012.026	1053.300	1032.457	1280.298			6106
5	5007	889.2552		835.031	782.474	942.428	1074.564			5007
6	4268					847.244	971.491			4268
7	3585						839.082			3585
8	2799						628.981			2799
9	2000						570.875			2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 32 (d): Telomere bands corresponding to Gel 8_4

Band Gel 8_5	A(M)	B	C	D	E	F	G	H	I	J(M)
1	21226	2115.236	1788.219	2056.811	2022.534	2033.896	2000	1942.229	2068.366	21226
2	8576	1815.532	1461.695	1691.540	1770.239	1806.382	1691.540	1824.729	839.732	8576
3	7427	1761.317		1461.695	1549.116		1632.757	1641.028		7427
4	6106	1641.028		848.557	1447.982		1519.827	1118.388		6106
5	5007	1549.116			810.173		989.445	951.897		5007
6	4268	1312.719					871.025	884.791		4268
7	3585	1164								3585
8	2799									2799
9	2000									2000
10	1900									1900
11	1584									1584
12	1515									1515
13	1429									1429
14	1164									1164
15	992									992
16	947									947
17	831									831
18	710									710
19	564									564
20	492									492

Table 32 (e): Telomere bands corresponding to Gel 8_5

The number of bands below 1500 bp were counted for each sample (8 reactions per sample) from the data shown above. The resultant number of bands less than 1500 bp for each sample are shown in the table below:

Patient ID/Sample number	Bands < 1500 bp
S1	11
S2	23
S3	8
S4	8
S5	15
S6	27
S7	11
S8	10
S9	25
S10	26
S11	22
S12	16
S13	11
S14	16
S15	19
S16	23
S17	19
S18	22
S19	30
S 20	19
S 21	31
S 22	23
S 23	32
S24	18
S25	12
S26	16
S27	15
S28	18

S29	26
S30	22

Table 33: Number of telomere bands below 1500 bp for each sample

These were then calibrated based on the initial PCR DNA template concentration and presented as the number of telomeres below 1500 bp per genome equivalent of template DNA.

B. Graph showing mean telomere length (with 95% confidence interval) by gender

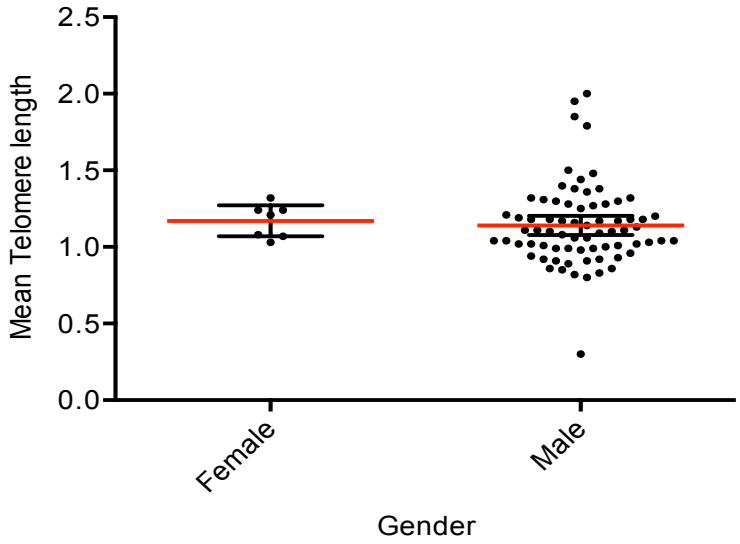

Figure 32: Mean telomere length (with 95% confidence intervals) by gender

C: Telomere associated SNPs

CHR	SNP	BP	N	P
10	rs9415039	73192704	1481	2.58x10-6
1	rs2801201	204726496	1474	8.02x10-6
3	rs12696304	170963963	1487	9.73x10-6
8	rs2167801	107563248	1485	1.17x10-5
3	rs7625734	170991616	1486	1.25x10-5
1	rs12083865	173962432	1484	1.32x10-5
3	rs9831661	171011232	1484	1.38x10-5
9	rs2680198	107051048	1481	1.41x10-5
3	rs10936601	171011152	1473	1.43x10-5
10	rs7090665	73713808	1485	1.73x10-5
12	rs7133043	66226912	1481	1.83x10-5
6	rs10806605	69569072	1483	2.35x10-5
2	rs624895	12779844	1485	2.48x10-5
13	rs1513031	103514088	1487	2.65x10-5
2	rs477599	12784768	1487	2.88x10-5
11	rs7110007	75679600	1487	2.93x10-5
3	rs7372527	46064344	1486	3.12x10-5
10	rs1149692	71386160	1478	3.13x10-5
5	rs9327948	105095600	1484	3.67x10-5
10	rs4418740	87471960	1486	3.91x10-5
13	rs1849450	103512256	1487	4.06x10-5
14	rs2268433	89936096	1473	4.24x10-5
13	rs9586352	103464672	1483	4.51x10-5
2	rs654004	12777856	1482	4.64x10-5
10	rs1014637	132419824	1480	4.72x10-5
12	rs1879405	117485592	1426	4.72x10-5
18	rs2298628	45709920	1483	4.82x10-5
8	rs13258014	4152868	1487	4.85x10-5

Table 34: List of top SNPs (P<1x10-5) associating with telomere length in the BHF-FHS cohort

{SNPs are given along with chromosomal (CHR) and base pair (bp) positions (estimated from HapMap build35). The number of individuals (N) included in the analysis for each SNP alongwith the P-value of the association are shown.}

CHR	SNP	BP	N	P
15	rs8032144	96175496	1421	2.28x10-6
1	rs12130746	212361952	1415	2.40x10-6
12	rs7960152	99078240	1322	2.58x10-6
9	rs10817041	110416280	1420	4.37x10-6
16	rs2052873	70062800	1421	4.43x10-6
9	rs10817042	110436016	1419	1.22x10-5
13	rs17073170	28764736	1419	1.26x10-5
16	rs16973260	70092352	1415	1.73x10-5
16	rs16972808	70036216	1422	1.78x10-5
12	rs4759709	129526352	1418	2.06x10-5
18	rs1876978	64418208	1412	3.37x10-5
7	rs2160059	10802988	1422	3.54x10-5
1	rs1325939	119296008	1419	3.64x10-5
7	rs17163856	10791039	1420	3.66x10-5
16	rs12922830	70004768	1415	3.81x10-5
7	rs6954256	10886998	1418	4.48x10-5
5	rs13354399	153169632	1421	4.66x10-5
10	rs11011867	20534772	1420	4.96x10-5

Table 35: List of top SNPs (P<1x10-5) associating with telomere length in the UKBS cohort

{SNPs are given along with chromosomal (CHR) and base pair (bp) positions (estimated from HapMap build35). The number of individuals (N) included in the analysis for each SNP alongwith the P-value of the association are shown.}