The Influence of Biological Ageing in the Pathogenesis of Colorectal Cancer

Fraser Maxwell
BSc (Hons) MBChB (Hons)

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University of Glasgow

College of Medical, Veterinary & Life Sciences

Department of Surgery

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SUMMARY

Despite improvements in risk factor awareness, diagnosis and enhanced management strategies, the incidence and five year survival of colorectal cancer, has remained largely unchanged over the last twenty years. As with many epithelial cancers, a preponderance of new colorectal cancer diagnoses occur in the over sixty five age group, making chronological age a strong risk factor. Given this indelible link between ageing and cancer in general, genetic pathways which are implicated in one process could influence the other. Thus, an understanding of the biology of ageing and factors which regulate it may provide insight into cancer pathogenesis.

Telomeres are nucleo-protein complexes sited at the ends of all chromosomes and have a critical function in the protection of the genome. Telomeres are implicated in the ageing process as a result of the inadequacies of the DNA replication machinery in somatic cells meaning that a section of telomeric DNA sequence is lost during each round of cell division, thus telomere length reduces with age and is a putative marker of biological ageing. Control of telomere length is complex and involves interplay between a number of genetic and environmental factors, of which oxidative stress is particularly important. However, critically short and hence dysfunctional telomeres have been implicated in cancer development through an inability to maintain genomic intergrity and an effect on senescence.

Telomeres play an integral role in the sensing and repair of DNA damage, however, cells must possess a finely tuned mechanism through which they can sense DNA damage and initiate a response. This usually involves the activation of cell cycle checkpoints, either temporarily to allow repair, or on an irreversible basis to prevent the clonal expansion of cells with deleterious mutations. If the damage is deemed irrepairable apoptotic pathways are initiated. The sirtuins are a group of genes first discovered and shown to control longevity in *saccharomyces cerevisiae*. Intense work has defined seven mammalian homologs termed SIRT1-7 which vary in their sub-cellular localisation, and have critical cellular functions ranging from the control of apoptosis, mitochondrial biogenesis, glucose and lipid metabolism, maintenance of genomic integrity and cell cycle control. Given these functions it is therefore no surprise that aberrancy of sirtuin expression is implicated in ageing and its commonly related diseases, particularly cancer. The aim of this study was

therefore, to determine if patients with colorectal cancer display aberrancy of ageing related factors, namely telomere biology and sirtuin expression.

This study was undertaken using two sources of material for investigation. Quantitative-PCR was utilised to measure telomere length in the peripheral blood leucocytes of 64 colorectal cancer patients and 1348 controls. In addition, telomere length was similarly measured in colorectal cancer tumour and normal adjacent tissue. Telomere length was then correlated with a number of clinical and pathological parameters to determine diagnostic or prognostic utility. Furthermore, an attempt was made to establish whether telomere lengths were reflected in circulating mediators of inflammation and redox control factors, including fetuin-A a circulating modulator of calcium homeostasis. Sirtuin relative transcriptional expression (SIRT1-7) was then measured in the tumour and normal tissue samples. Clinically relevant information was derived by analysing the SIRT1-7 transcriptional data in terms of clinico-pathological, inflammatory and outcome variables. Finally, sirtuin expression was correlated with other factors known to be involved with biological ageing to determine any potential association.

Colorectal cancer patients had significantly shorter telomeres in their peripheral blood leucocytes (adjusted mean RelT/S=0.61) compared with chronologically older controls (mean age 75, adjusted mean RelT/S=0.70) (ANCOVA, p=0.004), indicating colorectal cancer patients were biologically older than their control counterparts. In addition, telomere length in tumour tissue (median=0.43, IQR=0.40) was significantly shorter than adjacent normal tissue (median=0.65, IQR=0.28) (p=0.004). Patients with low plasma fetuin-A levels were shown to have significantly shorter telomeres (p=0.041) and patients with rectal tumours had significantly higher levels of fetuin-A than those with colonic tumours (p=0.045). There was no correlation between telomere length and other redox factors, namely anti-oxidant vitamins, micronutrients and divalent cations. There was, however, a significant association between telomere length and systemic inflammation as determined by the neutrophil to lymphocyte ratio.

SIRT 1-7 were differentially expressed between tumour and normal tissue, with significant attenuation evident in tumour samples when compared with normal tissue (p<0.0001 except SIRT2 p=0.003). SIRT2 (p=0.021) and SIRT4 (p=0.027) expression in tumour samples, was significantly associated with anatomical tumour site and pathologically determined nodal status respectively. Whilst, SIRT3 expression in normal tissue correlated

with pro-inflammatory status, indicated by higher serum CRP levels. Finally, there was a significant inverse relationship with colorectal cancer tissue telomere length and SIRT3. When overall survival was considered, Kaplan-Maier analysis revealed a significant difference in survival in relation to SIRT4 expression levels.

We have observed that patients with colorectal cancer display clear evidence of telomere attrition compared with controls. This is congruent with accelerated biological ageing in the pathogenesis of colorectal cancer and indicates cancer patients have ‘more miles on the clock’. An imbalance in redox control mechanisms and calcium homeostasis may be a contributing factor to telomere dynamics in these patients. The demonstration of attenuated sirtuin expression in colorectal cancer suggests a role as potential tumour suppressors and provides further evidence implicating biological ageing in the oncogenic process. Furthermore, plasma fetuin-A and tissue SIRT2 expression levels can be used to distinguish between colon and rectal cancers, providing further information regarding the molecular characteristics of these tumours. Telomere biology and the sirtuins could both play a pivotal role in the MTR (Mitochondria Telomere Ribosome biogenesis) paradigm, aberrancy of which could explain the apparent link between biological ageing and cancer. Enhancement of the understanding of the determinants of telomere length could mean that manipulation could lead to reduced colorectal cancer risk at the population level. In addition, the data provided in this thesis strengthens the evidence base which suggests that targeting individual sirtuins could be a future chemotherapeutic strategy, or indeed prove useful as markers of prognosis.

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PUBLICATIONS

Journal

Maxwell, F., McGlynn, L.M., Muir, H.C., et al. (2011). Telomere attrition and decreased fetuin-a levels indicate accelerated biological aging and are implicated in the pathogenesis of colorectal cancer. *Clin.Cancer Res.* **17(17),** 5573-5581.

Sebastian, C., Zwaans, B., Silberman, D.M. et al. (2012). The histone deacetylase SIRT6 is a novel tumour suppressor that controls cancer metabolism. *Cell* **151(6),** 1185-1199

Oral Presentations

The Influence of Biological Ageing in the Pathogenesis of Colorectal Cancer, Section of Epigenetics, MVLS, University of Glasgow, May 2012

Accelerated Telomere Attrition in Patients with Colorectal Carcinoma, submitted for e-poster of distinction, ASGBI, Liverpool, April 2010

Potential Role of Accelerated Biological Ageing and Antioxidant Status in Colorectal Cancer, WOSSA Autumn Meeting, Glasgow, Oct 2009

Poster Presentations

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Factors Involved in Biological Ageing Can Differentiate Between Colon and Rectal Cancers, ASGBI, Bournemouth, April 2011

Potential Role of Biological Ageing in Colorectal Cancer: How Many Miles on the Clock? Digestive Disease Week, New Orleans, USA, May 2010

Altered Sirtuin Expression in Colorectal Cancer, NCRI Conference, Birmingham, Oct 2009

Influence of Antioxidant Status and Biological Ageing in Patients with Colorectal Cancer, NCRI Conference, Birmingham, Oct 2009

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Authors Declaration

I declare that all work and analysis was carried out solely by me, except where indicated in the relevant sections of this thesis. In addition, statistical adjustment of the telomere data was performed with kind help from Dr Tony Robertson, MRC, Public Health Unit. Technical expertise in the analysis of anti-oxidant vitamin, micronutrient and divalent cation levels was provided and performed by members of the laboratory of Dr Dhinesh Talwar, Department of Biochemistry, Glasgow Royal Infirmary. The inflammatory cytokine assay was performed by Fiona Breckenridge (medical student).

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Abbreviations

Abf1	Autonomously replicating sequence binding factor 1
APC	Adenomatous Polyposis Coli
ATM	Ataxia Telangectasia Mutated
Bax	Bcl-2 associated X-protein
Bcl	B-cell Lymphoma
COPD	Chronic Obstructive Pulmonary Disease
CobB	Cobalamin coenzyme B12
DAF-16	Abnormal DAuer Formation-16
DDR	DNA Damage Response
FHL2	Four and a Half LIM 2
FISH	Fluorescence *in situ* hybridisation
FOXO	FOrkhead boX O
HIC1	Hypermethylated In Cancer 1
HIF-1α	Hypoxia inducible Factor-1α
H	Histone
HML	Homothallic Left
HDFs	Human Diploid Fibroblasts
IGF-1	Insulin like Growth Factor 1
HMR	Homothallic Right
KRAS	Kirsten Rate SArcoma virus
MAPK	Mitogen-activated protein kinase
MDC1	Mediator of DNA damage Checkpoint 1
MEF	Mouse Embryonic Fibroblast
MyoD	Myogenic Differentiation
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NAD^+	Nicotinamide Adenine Dinucleotide
NAMPT	NicotinAMide PhosphoribosylTransferase
Net1	Nucleolar silencing Establishing factor and Telophase regulator 1
NOXA	NADPH oxidase activator
PCAF	P300/CBP associated factor
PGC-1α	Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha
PI3-k	Phosphatidylinositol-3 kinase
PPARγ	Peroxisome proliferator-activated receptor Gamma
PUMA	P53 Up-regulated Modulator of Apoptosis
Rap1	Repressor activator protein 1
RENT	Regulator of Nucleolar silencing and Telophase exit
ROS	Reactive Oxygen Species
rDNA	Ribsosomal DNA
SOD	Superoxide dismutase
SNP	Single Nucleotide Polymorphism
SUN1	Sad1/UNC-84 homology 1
Wnt	Wingless-related MMTV integrated site

CHAPTER 1: INTRODUCTION

1.1 Ageing

Interest in the field of ageing has increased exponentially in the last few decades. The reasons for this are multi-factorial and include intellectual curiosity, significant observations stimulating new and more complex discoveries, and probably most importantly the increase in the average human lifespan as a result of 'epidemiological transition'. This transition has occurred in two stages the first of which resulted from improvements in public health and the development of antibiotics in the middle of the last century, along with a reduction in cardiovascular disease in the 1970's and 80's (Omran 1971). This increase in population longevity has major implications for public health and global expenditure on ageing related maladies including cancer. Therefore, improvements in the knowledge base surrounding the ageing process could improve our ability to predict further fluctuations in lifespan, whilst identifying key determinants of lifespan could lead to the development of targeted interventions in some of the major pathological conditions inexorably linked to the ageing process, particularly cancer. It has been apparent from the earliest days of ageing research, that ageing is a complex multi-factorial process determined by a number of different extrinsic (environmental) and intrinsic (genetic) mechanisms which control the rate of ageing as well as the phenotype.

Ageing itself has several definitions which broadly converge on the concept of increase in molecular chaos over time manifest as a detrimental change in phenotype culminating in an exponential increase in the likelihood of mortality (Shiels 1999). The concept of increase in death rate with age was first proposed by Gompertz (1825). Two main concepts emerge from this broad definition:

Chronological age; defined as the time elapsed since birth

Biological age; characterises the general condition of an individual at a certain time of his chronological age, which is marked by physical, psychical and social characteristics (Ries & Pöthig 1984).

Whilst this definition of biological ageing concerns the individual as a whole it can be scaled down to the cellular level, where it could describe the general condition of the molecular machinery maintaining cellular homeostasis. When the two concepts of ageing

are compared they rarely equate with biological age subject to a variety of intrinsic and extrinsic forces which determine the level of damage accrued over time and the ability to counterbalance it (Karasik *et al*, 2005). The intrinsic forces which contribute to biological ageing include genetics, methods of maintaining genomic stability, epigenetic phenomena such as methylation or accumulation of anomalous proteins with the ability to resist normal degradation pathways (Kirkwood 2008). Extrinsic forces comprise a variety of environmental factors including nutrition, exercise, socioeconomic status and smoking that act to alter the rate of biological ageing. Aberrancy of any of these broad intrinsic factors or manipulation of extrinsic factors could therefore alter the course of biological ageing in a positive (deceleration) or negative (acceleration) fashion and provides the basic framework for ageing research ranging from the population to the molecular level.

1.1.1 Ageing as an Evolutionary Concept

Evolutionary concepts of ageing were devised in an attempt to answer fundamental questions such as ‘Why do we age’ and ‘Why do we live as long as we do’?

1.1.1.1 Accumulated Mutations Theory

Medawar first proposed this theory in 1952. The central theme is the idea that detrimental, late acting mutations may accumulate in the population and ultimately lead to growth arrest and pathology (Medawar 1952). The basis for these observations was the expansion of the knowledge surrounding Huntington’s disease. In the present day when there is relatively little risk of extrinsic pressures on mortality such as that from infectious disease or predation there is no real selection pressure to remove these late acting deleterious mutations

1.1.1.2 Antagonistic Pleiotropy Theory

This theory, also called the ‘trade-off theory’ espouses that genes exist which can have beneficial effects earlier in life but harmful effects later. The aim of optimal health earlier in life is to allow reproduction (Williams 1957). However, this theory has limited application in the modern human population where limited extrinsic pressures prevent early mortality thereby allowing the late onset deleterious effects to manifest. A physiological example of this theory would be the requirement of the prostate gland on

androgens for normal function, however later in life androgen exposure may contribute to the risk of prostate cancer (Imamoto *et al*, 2008).

1.1.1.3 Disposable Soma Theory

This theory argues that the investment in durability and maintenance of somatic tissues are predicted to keep the body in good repair through the normal expectation of life in the wild with some degree of reserve capacity (Kirkwood 1977, Kirkwood 2008). Essentially, the somatic organism is effectively maintained to allow reproductive success and once this has occurred becomes disposable, manifesting in ageing phenotypes. However, the reproductive tissues must be maintained to a level that allows accurate replication and passage of genetic material across a number of generations. This theory explains inter-species variation in lifespan. The higher the extrinsic mortality rate of a species, the less energy should be invested in somatic maintenance and more towards reproduction, culminating in shortened lifespan (Kirkwood 2002).

The previous two theories state the evolution of ageing results from gene action. In contrast, the disposable soma theory predicts that ageing evolves by genetic neglect, resulting in accumulation of molecular damage and eventually giving rise to age-related frailty and disease.

1.1.2 Molecular & Cellular Mechanisms of Ageing

Within the framework of the evolutionary theories of ageing described above there are a number of potential mechanistic pathways contributing to the decline in molecular and cellular viability associated with the ageing phenotype. These are varied and not mutually exclusive hence it is unlikely that any one acts independently (Table 1.1).

Table 1.1: Table summarising the key features of the main molecular and cellular mechanisms of ageing.

Concept	Key Features	References
Genetics	• MZ twin lifespan more concordant than DZ.	(vB Hjelmborg *et al*, 2006)
	• Implicated genes include insulin/IGF1, FOXO and APOE, also chromosome 11p15.5.	(Puca *et al*, 2001, Starr *et al*, 2008, Flachsbart *et al*, 2009, Pawlikowska *et al*, 2009)
Senescence	• Initiated by the observation of cessation of division by cells in culture after a certain number of replication cycles, termed the Hayflick limit.	(Hayflick & Moorhead 1961)
	• Progressed by subsequent discovery that telomere attrition is a major driver of senescence and telomere length shortens with chronological age as a result of the end replication problem.	(Olovnikov 1973, Aubert& Lansdorp 2008)
	• Senescent cells alter organ function and may contribute to the ageing phenotype.	(Campisi 2005, Campisi& d'Adda di Fagagna 2007)
Genomic Instability	• Maintenance of genomic integrity essential to prevent development of deleterious mutations which could contribute to the ageing process.	(Baute & Depicker 2008)
	• Checkpoint proteins (Chk1 & Chk2) monitor the genome and can halt cell cycle progression at either the G_1/S or G_2/M transition points, to allow the damage to be assessed and the requisite response elicited (repair, apoptosis or senescence).	(Stracker *et al*, 2009)
	• DSB repaired via ATM and Chk2 pathway, single strand breaks via ATR and Chk1, downstream targets of both pathways include p53 and DNA repair proteins e.g. Ku.	(Baute & Depicker 2008, Smith *et al*, 2010, Warmerdam *et al*, 2010)
Cumulative oxidative Burden	• Free radical theory of ageing states that longevity is limited as a result of cumulative damage to DNA and other macromolecules by reactive oxygen species (ROS). It is theorised that long-lived individuals have an augmented stress resistance capacity or produce less ROS.	(Harman 1956)
	• Over-expression of SOD in model organisms can increase longevity.	(Moskovitz *et al*, 2001, Salmon *et al*, 2009)
Mitochondrial theory	• Mitochondria are the main source of ROS in the cell and as such play a key role in the balance between oxidative damage and repair. Dysfunctional mitochondrial metabolic processes produce an excess of ROS via mutated mitochondrial DNA amongst other mechanisms.	(Edgar *et al*, 2009, Edgar & Trifunovic 2009)
	• Murine models of mutated mitochondrial DNA display evidence of accelerated ageing.	
Nutrient Sensing pathways	• Nutrient sensing is a key evolutionary conserved process which stimulates homeostatic modification to gain a survival advantage for organisms at times of low nutrient intake, allowing the potential for reproduction to be maintained. Two main pathways include:	
	1. Insuin/IGF1- proliferation, protein synthesis, and the capacity to resist stress. These functions are facilitated through a complex cascade of kinases	(Salminen & Kaarniranta 2010)

	such as the PI3-K/Akt/FOXO pathway 2. mTOR- integrates cellular response with nutrient and energy levels through interaction with AMPK, responses include protein synthesis and ribosome biogenesis, autophagy, and mitochondrial activity. mTOR inhibition in murine models	(Harrison *et al*, 2009, Zoncu *et al*, 2011)
Epigenetics	• Epigenetic phenomena are modifications to genes such that expression is altered without changes to the DNA sequence. This leads to aberrancy of gene expression most commonly resulting from chromatin remodelling due to processes such as DNA methylation and histone modification by methylation and acetylation. • The pattern of epigenetic modifications and chromatin structure is dynamic and varies throughout the lifespan of an organism- hypermethylation leads to suppression of expression of target genes with hypomethylation augmenting responsiveness.	(Gravina& Vijg 2010)
Stem Cell	• Stem cells in adult tissues are responsible for the maintenance of function and structural integrity, due to differentiation or replenishment. Attenuation of stem cell function may contribute to the decline in tissue integrity and organ dysfunction associated with ageing. • Relationship with altered telomere biology and reduced capacity to facilitate DNA repair.	(Charville & Rando 2011) (Flores& Blasco 2010, Rube *et al*, 2011)

Calorie Restriction can Increase Lifespan

Caloric restriction (CR) was first demonstrated to influence longevity in rodent studies performed in the 1930's, since then a plethora of studies have replicated these findings in a number of different model systems from yeast to non-human primates (Kaeberlein *et al*, 2004, Colman *et al*, 2009). CR stimulates a number of molecular adaptations which lead to robust changes in whole organism patho-physiology. Major changes induced by CR include reduction in body weight, body temperature and metabolic rate (Redman & Ravussin 2011, Rochon *et al*, 2011). Whilst endocrinological responses include improved glucose tolerance and insulin sensitivity, equivalent serum IGF-1, reduced T_3 and increased cortisol (Redman *et al*, 2008).

1.2 Ageing and Cancer

1.2.1 Trajectories of Ageing

A recurring theme through the evolutionary concepts of ageing is one of degeneration in function as time progresses. Three trajectories of ageing have been described (Weinert & Timiras 2003).

1. First, ageing characterised by disease and disability.
2. Second, 'usual' ageing characterised by some decline in function but no overt pathology.
3. Third, 'successful' ageing where there is little or no evidence of pathology and no decline in function.

Ageing is deemed successful when an individual is free from disease and disability, has high cognitive and physical functioning, and engages in social and productive activities. Clearly a spectrum exists across the range of ageing phenotypes. As our adaptive and homeostatic mechanisms decline in function so the probability of disease and pathology increases. Although disease is not considered a prerequisite in the process of ageing a number of pathologies occur at a much higher frequency in older individuals. One of the most obvious examples is cancer.

1.2.2 Common Mechanisms in Ageing & Cancer

Cancer is to all intent and purposes a disease of ageing with 77% of all new diagnoses made in patients over the age of 55. This is mainly due to an excess of epithelial cancers, particularly breast, lung, colon and prostate, as opposed to those of mesenchymal or haematopoietic origin. For this reason age is considered the most potent of all carcinogens. The lack of a concomitant increase in tumours of diverse types with age suggests a complex relationship (DePinho 2000).

Age-related diseases are generally thought to result from a decline in tissue structure and function, an overall degenerative process. However, despite being considered a disease of ageing, cancer is paradoxically a gain of function disease, with cells acquiring the ability to hyper-proliferate and ultimately migrate. Mammalian species including humans have evolved the ability to renew somatic tissues which evidently confers an advantage, with the ability to repair damaged tissue to restore function. This ability, however, also gives rise to an elevated cancer risk as DNA replication is not fullproof thus potentially increasing the risk of oncogenic mutations. In addition to mutational load, cancer development also requires specific gene silencing through epigenetic regulation and a permissive environment in which cellular growth is encouraged. In order to prevent cancer formation a number of tumour suppressive mechanisms have evolved.

1.2.2.1 Tumour Suppressor Mechanisms

Tumour suppressors can be broadly categorised into two distinct groups. Caretaker tumour suppressors act on the genome to prevent or repair DNA damage. Mutations are therefore suppressed and risk of neoplastic transformation reduced. Examples of caretaker tumour suppressor genes include BRCA1/BRCA2, ATM and deleted in breast cancer-1. As deleterious events are reduced by caretaker tumour suppressors longevity is increased (van Heemst *et al,* 2007).

The second main group of tumour suppressors are termed gatekeepers. These function to eliminate potentially cancerous cells by initiating senescence, thereby preventing further growth or division, or stimulating apoptosis. The gatekeeper tumour suppressor which has come under the most scrutiny is p53, due to its pervasive presence in many of the pathways controlling both cancer and ageing it deserves special mention.

p53- a gatekeeper tumour suppressor

p53 is a multi-functional transcription factor which regulates the transcription of a huge number of genes related to cellular processes such as response to stress, cell cycle checkpoint regulation, DNA repair and angiogenesis. Clearly these processes are fundamental to the initiation and progression of oncogenesis and it is therefore no surprise that p53 is mutated in almost 60% of human cancers (Vogelstein *et al,* 2000). The tumour suppressor effect of p53 arises as a result of activation of the ATM/ARF pathway by DNA damage or alternatively critically short telomeres. This propagates a response in which human double minute-2 (HDM2) is sequestered and its negative feedback relationship with

p53 reversed. The resulting increase in p53 expression facilitates either senescence or apoptosis. The decision as to which fate a cell is subjected to is not fully understood and likely to result from the damage stimulus and tissue type (Zuckerman *et al,* 2009).

Recently p53 has been implicated as a regulator of the ageing process, in addition to its well described effect as a tumour suppressor. One might expect that as a potent tumour suppressor the effect on longevity would be simply that lifespan is extended as a result of a reduced incidence of cancer. However, p53 gain of function mutant mice exhibit an extraordinary resistance to cancer but died prematurely, exhibiting signs of accelerated ageing. Thus p53 over-expression results in a chronic engagement of DNA damage signalling which results in accelerated ageing phenotype and therefore has a marked pro-ageing effect. (Tyner *et al,* 2002, Maier *et al,* 2004). Further complicating matters is the possibility that gatekeeper tumour suppressor genes including p53 could exert an antagonistic pleiotropic effect. In young individuals these genes have a beneficial effect by preventing the development of cancer. However, as lifespan progresses this effect can become deleterious and promote the ageing phenotype. In evolutionary terms because we are no longer subject to extrinsic hazards which curtail our lifespan (predation, infection etc) we now live longer, and as such are genetically exposed to the delayed detrimental effects of genes which are beneficial to younger organisms (Campisi 2005).

It is clear the relationship between ageing and cancer is not a simple one to rationalise and that multiple layers of pathways controlling both processes exist. The initial underlying stimulus for both processes appears to be similar with disruption of genomic integrity, either within the genome in general or at the telomere. The response to this damage is integrated through the many of the same pathways such as p53, ATM/ARF and nutrient sensing pathways such as mTOR or the sirtuins. It is clear however, that the end phenotypes are distinct and that regulation of these pathways at the genetic, epigenetic and post-transcriptional level is responsible for these differences.

1.2.3 Colorectal Cancer

Colorectal cancer is the third most common cancer in the UK with 41,000 new cases diagnosed in 2009, and accounts for 14% of all new cases of cancer in general. Incidence

rates are approximately 75 / 100,000 in men and 59 / 100,000 in women, this difference is explained by a higher incidence of rectal cancer in men. Pertinent to this thesis is the fact that 72% of bowel cancers develop over the age of 65, making age a strong risk factor for the disease. This age related increase peaks between 75 and 80 where it remains at 500 new cases / 100,000 in men and 338 / 100,000 in women. Incidence rates have remained on the whole static over the last twenty years, with survival rates doubling. However, overall five year survival is relatively poor at only 50% in both males and females (Cancer Research UK 2010). A short discussion of some of the common issues and terms with regards to CRC and pertinent to this thesis will now follow.

1.2.3.1 Diagnosis and Staging

Currently in the UK the pathways through which patients with CRC reach a diagnosis are altering. In the past symptomatic patients would be referred by their primary care physician generally with symptoms such as altered bowel habit, rectal bleeding, weight loss or local symptoms such as tenesmus. In addition to this group of patients there are a constant proportion of patients with CRC who present as an emergency with acute symptoms in relation to disease progression leading to bowel obstruction, perforation or acute haemorrhage (Crozier *et al,* 2009). The presentation of patients with symptoms whether emergent or otherwise has historically meant that a large proportion of patients present at a late stage when the chances of curative intervention are dramatically diminished. In an attempt to combat this phenomenon the National Bowel Screening Programme was recently implemented. This was done so on the basis of a number of trials which have shown that bowel screening for CRC by faecal occult blood testing can reduce mortality, with the most recently published UK trial showing a reduction in CRC specific mortality of 13% (Scholefield *et al,* 2011). Based on this, and similar evidence the National Bowel Screening programme was initiated, which in Scotland involves an invitation from the age of 50-74 to submit a biennial faecal occult blood test. Should this prove positive patients are then invited to undergo a colonoscopy to visualise the mucosa of the colo-rectum, biopsy any abnormal areas or perform polypectomy.

Staging of colorectal cancer is a fundamentally important process as the pre-operative staging of the disease will determine the treatment modality and the prognosis on an individual basis. For patients with colon cancer this involves a computed tomography (CT) scan to determine spread of disease to distant organs and some indication of local invasion.

Patients diagnosed with rectal cancer will undergo an additional MRI scan of the pelvis to determine the extent of local invasion. The TNM and Dukes classification are the most commonly used staging systems (Table 1.2). Both of these are pathologically determined therefore full assessment can only be made after surgical resection and pathological analysis of the tumour specimen.

Table 1.2: Table summarising the components of the TNM and modified Dukes classification of colorectal cancer.

TNM Classification				**Modified Dukes Classification**	
Stages	**T**	**N**	**M**	**Stages**	
Stage 0	Tis	N0	M0		Disease confined to the bowel wall or adjacent structures
Stage I	T1	N0	M0	A	
	T2	N0	M0	B1	
Stage II	T3	N0	M0	B2	
	T4	N0	M0	B2	
Stage III	T1,T2	N1 or N2	M0	C1	Node positive disease
	T3,T4	N1or N2	M0	C2	
Stage IV	Any T	Any N	M1	D	Metastatic disease

Details of T-stage given in Figure 1.9, Tis = in situ disease (not fully invasive), N1 = 1-3 positive lymph nodes, N2 = ≥ 4

1.2.3.2 Pathological Concerns

Tumour Site & Molecular Characteristics

As depicted in Figure 1.1 the spread of distribution of tumours throughout the colo-rectum favours a predilection for the left or distal region with a ratio of approximately two thirds to one third. Distal (left colon and rectal) cancers are generally defined as those distal to the splenic flexure, with proximal (right and transverse) cancers the converse.

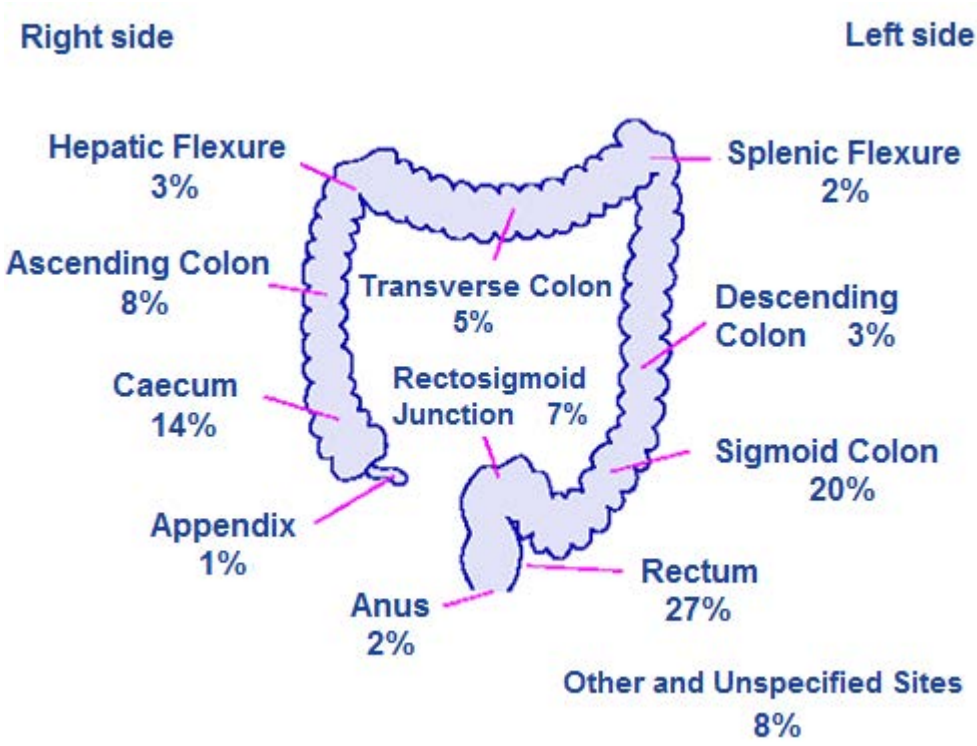

Figure 1.1: Distribution of tumour site throughout the colorectum (Cancer Research UK 2010).

Recent interest in tumour site has lead to the discovery that there are particular clinical and molecular characteristics associated with the site of the tumour, meaning that CRC is now considered a heterogeneous disease. Initial characterisation was made on the basis that colon and rectal cancers were distinct entities but it now seems that proximal versus distal cancer is a more appropriate differentiation, however as molecular characterisation evolves this may also change (Minoo *et al*, 2010). The fundamental molecular difference lies in the driver of oncogenesis with proximal tumours generally initiated by dysfunctional mismatch repair pathways resulting in microsatellite instability, poor differentiation, mucinous histology and lymphocytic infiltration. Furthermore, these tumours are more likely to be hereditary resulting from an inherited mutation of one of the mismatch repair genes, most commonly *MSH2*. Distal and rectal tumours are more likely to result from transformation within an adenomatous polyp via the adenoma-carcinoma sequence (discussed in due course), more likely to be microsatellite stable, have a higher frequency of *KRAS* mutation and more likely to be sporadic (Gordon 2007, Cunningham *et al*, 2010)

Adenoma-Carcinoma Sequence

This represents the proliferation of a single epithelial cell, to a benign polyp and sequentially on to a malignant neoplasm through a well defined series of genetic

mutational events (Figure 1.2). This was termed the adenoma-carcinoma sequence and characterised by the seminal work of Fearon and Vogelstein (Fearon & Vogelstein 1990). The clinical significance of the adenoma-sequence is that it takes approximately 7 years for normal epithelium to develop into a malignant lesion thus meaning that intervention to remove polyps should in theory prevent the development of invasive cancer, this provides the basic rationale behind the National Bowel Screening Programme (Gordon 2007).

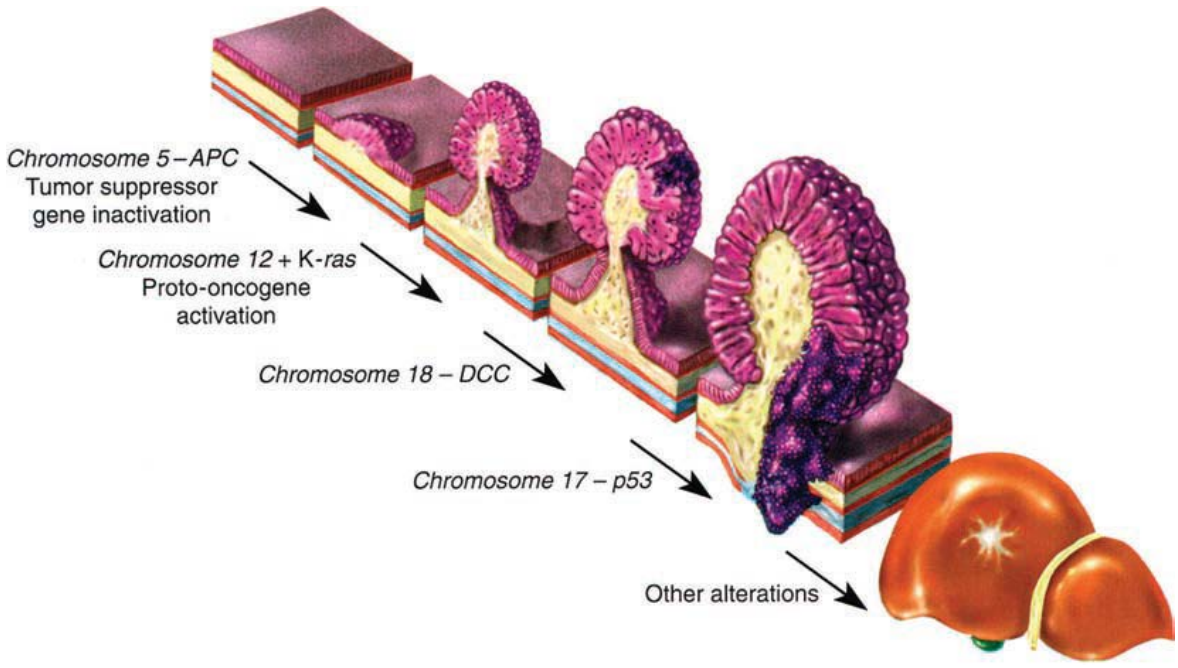

Figure 1.2: The sequence of genetic events and chromosomal site of progress through the adenoma-carcinoma sequence. Taken from Gordon (2007). DCC- deleted in colorectal cancer

1.2.3.3 Management Strategies

The principle behind the surgical management with curative intent of CRC is resection of the tumour with adequate resection margins and sufficient lymphadenectomy to allow accurate staging of the disease. Surgical management of rectal cancer involves total excision of the mesorectum (TME) and adequate distal resection margins, if the lesion is too close to the anal verge to allow anastamosis the sphincter complex is resected and an end colostomy formed. As mentioned previously patients with rectal cancer undergo an additional staging pelvic MRI to assess the degree of local invasion of the tumour (Figure 1.3). If on the basis of preoperative staging circumferential resection margins are deemed compromised patients are offered neoadjuvant therapy with either radiotherapy alone or in

combination with 5-fluorouracil based chemotherapy to downstage the disease and increase the likelihood of curative resection (Sebag-Montefiore *et al,* 2009, Cunningham *et al,* 2010). Generally patients who are lymph node positive (Dukes C) are offered post-operative adjuvant chemotherapy to reduce the likelihood of systemic recurrence. Depending on the functional status of the patient combinations of either platinum or 5-fluorouracil based chemotherapeutic regimens are employed (IMPACT Investigators 1995). Dukes B or node negative patients prove more complex as the benefits of chemotherapy are less well established with only small gains in survival (Quasar Collaborative Group *et al,* 2007). The discovery during staging of metastatic disease does not preclude surgical intervention as synchronous or staged resection of the primary tumour and liver resection can be undertaken (Moug *et al,* 2010).

A B C

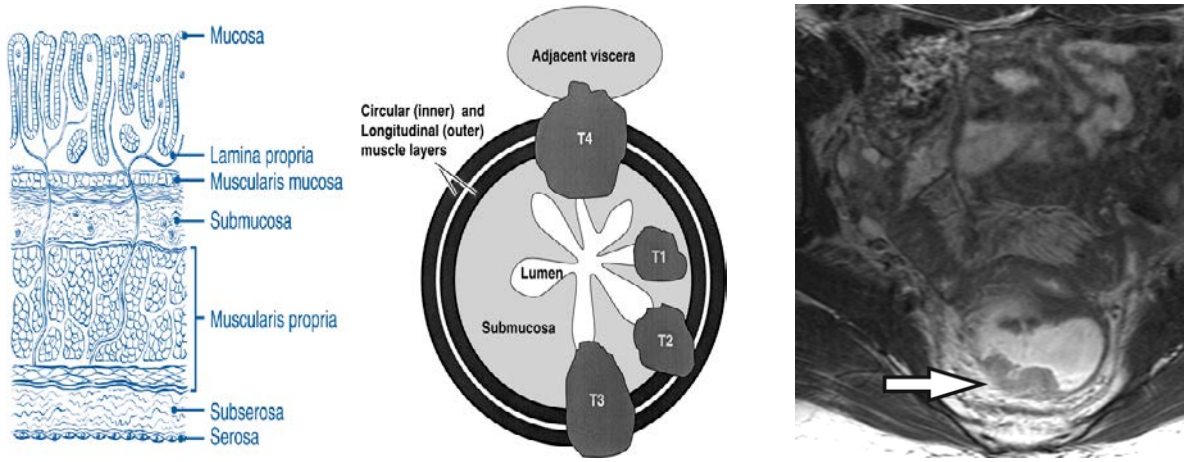

Figure 1.3: A Schematic representation of the layers of the colonic and rectal wall. B T-stage characterisation of colorectal cancer: T1- confined to submucosa, T2- extends beyond muscularis propria, T3- invades into subserosa T4- extends beyond subserosa and into adjacent structures. (Brown *et al,* 1999). C MRI representation of T3 rectal cancer, lesion extension into subserosa indicated by the arrow (Kim *et al,* 2004).

It is clear that there are a number of areas of colorectal cancer biology and management where ambiguities lie, and which would benefit from an expansion of current knowledge. Obvious areas include the further molecular characterisation of tumours from different sites in the colo-rectum and identification of patients who are deemed to have poorer prognosis and may benefit from alteration of traditional follow-up and treatment strategies.

The clear increase in risk of CRC with age could provide a platform from which to further investigate the link between biological ageing and CRC. This thesis looked specifically at the role of telomere biology and sirtuin expression, the remainder of this introduction will outline the background of these two important areas of ageing and cancer biology.

1.3 Telomere Biology

In recent years the body of work surrounding telomere biology has increased exponentially. Furthermore, as the intricacies of the functional processes controlled by the telomere complex become more apparent, the role of telomere biology in the ageing process becomes more defined. Altered telomere biology has now been associated with a variety of age related pathologies from heart disease to cancer. A discussion of the structure and function of the telomere complex, along with control mechanisms determining telomere length (TL) will now follow, as will the relationship between TL and cancer.

1.3.1 Telomere Structure

The term telomere was first coined by Herman Muller during his seminal work with fruit flies, from the Greek meaning of ‘end’- telos and ‘part- meros. Both he and Barbara McLintock working with maize proposed that chromosome ends possess special structures required for chromosome stability and that without them chromosomes would fuse leading to breakages (McClintock 1939).

1.3.1.1 Telomere DNA Structure

The basic structure of the telomere is that of a nucleo-protein complex consisting of telomeric DNA and a number of bound proteins which are vital for the regulation of TL and the functionality of the telomere. Telomeric DNA structure consists of tandem repeats of G-rich sequences (TTAGGG)n (Moyzis *et al*, 1988). Investigation of the telomere structure using electron microscopy revealed the presence of large loops. These loops were dependent on the 3’G-strand overhang, which were tucked back inside the double stranded DNA and termed t-loops. Further analysis revealed the presence of a further loop structure

termed the d-loop (Greider 1999) (Figure 1.4). This 'displacement' loop is formed by single stranded DNA invasion of a double stranded sequence and base pairs with one of the strands. In the telomere this sequence extends for approximately 100-200 base pairs with the TTAGGG G-strand base paired to the CCCTAA sequence (Griffith *et al*, 1999a). The function of this loop structure is to enhance the ability of the telomere to protect the chromosome end from being recognised as a DNA break. This is not hard to envisage given the now 'hidden' 3' overhang.

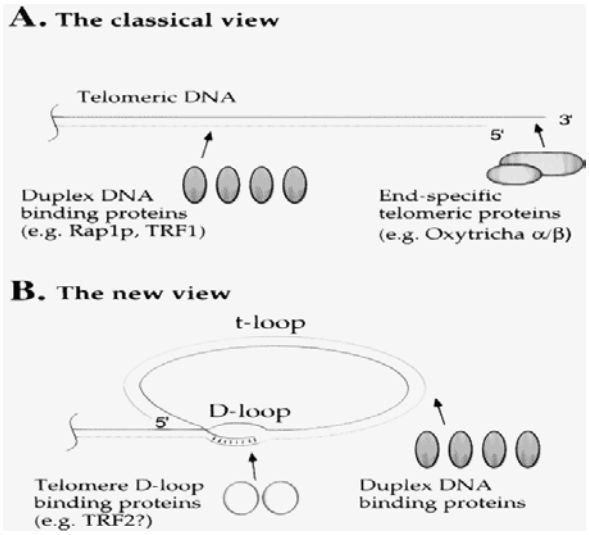

Figure 1.4: Demonstration of the classical and newer 'loop' structure of the telomere. The original proposition of the telomere structure was that of a linear double stranded structure with a 3' overhang on the G-strand and telomere-associated proteins positioned along its length. (Greider 1999).

1.3.1.2 Telomere Associated Proteins

Combined with the DNA structure of the telomere are a variety of proteins and enzymes, which confer structural integrity on the telomere, are responsible for elongation of telomeric DNA and aid in the functionality of the telomeric complex. Some of these proteins are permanently bound to the complex whereas others have a more dynamic relationship depending on cellular conditions such as stress and DNA damage. A schematic diagram of the most important telomere associated proteins is shown below (Figure 1.5).

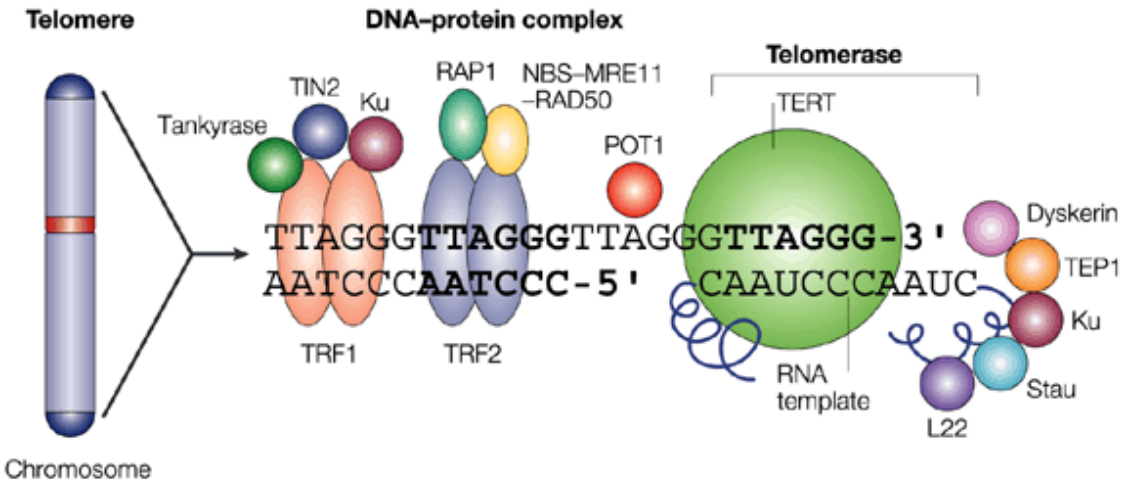

Figure 1.5: Telomeric structure, including the major associated proteins (Hodes *et al*, 2002).

Telomerase

Telomerase is a specialised unique enzyme which is responsible for the elongation of the telomeric TTAGGG repeat sequences. It was first described in *tetrahymena thermophilia* and is composed of three core subunits; the first is the reverse transcription protein termed TERT and encoded by the *hTERT* gene located on chromosome 5p.15.33. Secondly, the RNA template for the TTAGGG sequence termed TERC, the *hTERC (or hTR)* gene is positioned at 3q21-q28. Finally, the third subunit is defined on a species specific basis which in humans is dyskerin, this is required for accurate folding and structural stability of the telomerase complex (Greider & Blackburn 1987, Morin 1989, Cohen *et al*, 2007) (Figure 1.6).

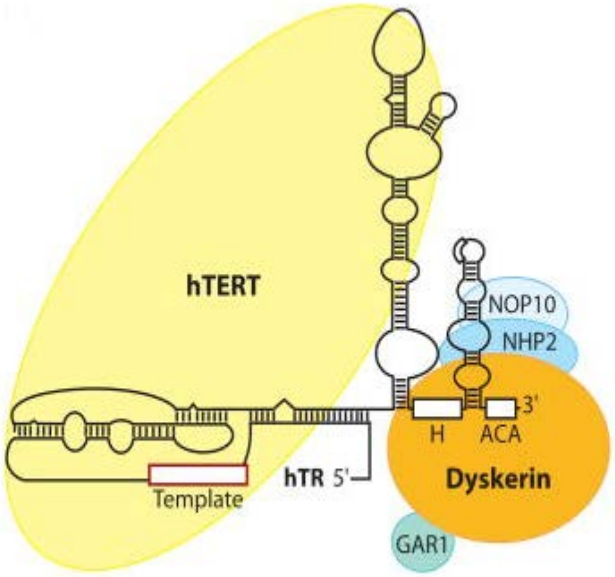

Figure 1.6: Diagram of the telomerase structure. NOP10, NHP2 and GAR1 are subunits of the dyskerin complex and are required for the correct processing of telomerase. hTR is interchangeable with hTERC. Adapted from (Armanios 2009)

Shelterin

Shelterin is a complex of six proteins composed of TRF1, TRF2, TIN2, Rap1, TPP1 and Pot1 (Figure 1.5), with all six proteins found in a single complex in fractionated nuclear extracts. TIN2 is considered to be the most important constituent of the complex due to its ability to tether TPP1/Pot1 to TRF1 and TRF2, whilst also linking TRF1 to TRF2 (de Lange 2005). Shelterin shows great specificity for the TTAGGG sequence of telomeric DNA by virtue of multiple recognition folds within the structure of TRF1, TRF2 and Pot1 (Court *et al,* 2005). Shelterin is thought to shape the telomeric structure by encouraging the formation of the t-loop, a function particularly dependent on TRF2, given its ability to remodel artificial telomeric substrate into loops *in vitro* (Stansel *et al,* 2001). The presence of shelterin and the t-loop configuration function together to form a 'cap' on the telomere which functions to protect the chromosome end. Shelterin also contributes to the maintenance of TL and the DNA damage response mechanism.

Dynamic Telomere Associated Proteins

This is a substantial group of proteins which transiently bind to the telomere and shelterin complex. Many of these proteins have a dual role in regulating the DNA damage response

in addition to contributing to the maintenance of optimal TL. Some of these proteins and their key functions are listed in the table below.

Table 1.3: Table listing some of the major transient telomere associated proteins with their binding targets and function.

Protein	Binding Site	Function
Tankyrase	TRF1	TRF1 down-regulation with resulting increased activity of telomerase and positive regulation of telomere length
DNA-PK	TRF1	Aids in creation of t-loops
DNA-PKcs	TRF2	Signals short telomeres as DNA damage
Ku70/86	dsDNA	Inhibits telomerase Recruits DNA-PKcs Recruits/stimulates WRN/MRN Regulates Atm
MRN Mre11 Rad50 NBS1	 TRF2 TRF2 TRF1/2	Modulates Atm t-loop stabilisation stimulates Mre11 activity Unpairing/opening t-loop
WRN	TRF2	Unwinds 3' loop
BLM	TRF1	Unwinds dsDNA
RPA	ssDNA	Prevents re-annealing
Atm	TRF2	DNA damage signalling
ERCC1	TRF2	Removes G-overhang
BRCA1	TRF1	Regulates transcription hTERT

DNA-PK, DNA-protein kinase; DNA-PKcs, DNA-protein kinase catalytic subunit; dsDNA, double stranded DNA; Mre11, meiotic recombination 11; NBS1, Nijmegen breakage syndrome 1; WRN, Werner syndrome; BLM, Bloom syndrome; RPA, Replication protein A; ssDNA, single stranded DNA; Atm, Ataxia-Telangiectasia-mutated; ERCC1, Excision repair cross-complementing 1; BRCA1, Breast cancer associated 1

1.3.2 Maintenance of Telomere Length

1.3.2.1 Telomere Dynamics

Maintenance of TL is critical to telomere functionality as upon reaching a critically short length a series of responses are elicited which can ultimately lead to the removal of the cell from the population. Therefore, a number of mechanisms have evolved to maintain TL at a

constant length. However, this is complicated by the fact that one of the key mechanisms for maintaining TL, the enzyme telomerase, is 'switched off' in somatic cells meaning that as they replicate TL shortens, this observation lead to the concept of TL reflecting the replicative capacity of the cell and functioning as a mitotic clock. This link with replicative capacity is not limited to the cellular level as TL also reduces with increasing chronological age, a process which can be altered by a number of intrinsic and extrinsic factors.

Maintenance of TL is a complex process which is reflected by the fact that TL is highly variable. Average TL in normal human population varies between 5-10Kb but this can vary on an individual basis between organ systems, cells and even between chromosomes. Variability within individuals was demonstrated by Takubo *et al* (2002) who showed that the longest telomeres were evident in the myocardium with the shortest in the liver and renal cortex. This may reflect the replicative and rejuvenative capacity of these organs, with non-replicative tissues retaining baseline TL (Takubo *et al,* 2002). The heterogeneity of TL has recently been partially characterised in a longitudinal study following 635 volunteers over a 5-10 year period. The rate of change in TL was highly variable with some individuals seemingly gaining in TL, however a shorter TL at baseline was translated into a more rapid loss of TL overall. The authors therefore conclude that factors other than the end-replication problem influence TL dynamics (Aviv *et al,* 2009).

1.3.2.2 End Replication Problem

The end replication problem suggests that TL reduces with every round of cell division until a critical length is reached, when signalling pathways are activated which remove the cell from the replicating population by either apoptosis or senescence. The end replication problem arises due to the uni-directional nature of DNA replication and the 5' to 3' function of DNA polymerase. This means that synthesis of the lagging strand must occur discontinuously with small RNA primers added a short distance ahead to elongate the DNA sequence via the formation of Okazaki fragments. However, when the replication fork reaches the end of the lagging strand a gap is left in the sequence once the RNA primer has been removed. This means DNA polymerase is unable to replicate the sequence, therefore a section of telomeric DNA is lost with subsequent replications (Levy *et al,* 1992). The concept of the number of repetitive sequences at the chromosome end determining the replicative capacity of somatic cells, as well as the telomeric repeat sequences acting as a protective buffer to upstream genes was put forward by Olovnikov

(1973). In addition, Olovnikov also suggested that cells from continually dividing tissues and organs required a mechanism by which to overcome the end replication problem. The discovery of telomerase and its ability to elongate telomeric DNA repeats solved the mystery of the end replication problem (Olovnikov 1973).

1.3.2.3 Role of Telomerase

Telomerase is considered prerequisite for any cell type which has the need to undergo continual division. Thus telomerase is expressed in over ninety percent of human tumours and is also constitutively expressed in germ-line cells and many types of human stem cells (Kim *et al,* 1994). Modest amounts of telomerase activity have been discovered in progenitor cells of skin, gut epithelium and bone marrow, reflecting the potential for renewal in these cell compartments (Artandi & DePinho 2010). Introduction of telomerase into telomerase null cells confers a survival advantage with telomerase expressing cells displaying longer TL, increased replicative capacity and reduced evidence of cellular senescence (Bodnar *et al,* 1998).

In vitro reconstitution experiments with single amino acid modified hTERT confirms it to be the catalytic component of the telomerase enzyme, and essential for telomerase function (Weinrich *et al,* 1997). Immortality of normal prostate cells in culture is achieved by c-myc mediated up-regulation of hTERT expression and results in stabilisation of TL (Gil *et al,* 2005). Factors essential for normal cell cycle checkpoints and maintenance of senescence such as p53 and $p16^{INK4A}$ can down-regulate hTERT expression (Kanaya *et al,* 2000, Bazarov *et al,* 2010). Conversely, growth factors known to stimulate cellular proliferation such as the viral oncogenes human papilloma virus and cytomegalovirus can up-regulate hTERT expression (Horikawa & Barrett 2003, Straat *et al,* 2009). Variation at the TERC gene has also been shown to influence TL. Genome wide association analyses have revealed a locus at 3q26 which correlates with mean leucocyte TL, a locus which includes the TERC gene (Codd *et al,* 2010).

1.3.2.4 Components of Shelterin Influence Telomere Length

The shelterin complex as a whole and its constituents influence TL in a number of ways. TRF1 and TRF2 are negative regulators of TL acting indirectly to control the access of telomerase to the telomere. This is performed by facilitation of an isometric change in the telomere to *cis* configuration, by doing so the telomere changes from an open to a closed state preventing the access of telomerase and an inability to elongate TL if required (De

Boeck *et al,* 2009). It is postulated that TRF1 and TRF2 effectively sense TL, as the longer the telomere the more abundant the levels of TRF1 and TRF2, with very short telomeres unable to bind enough TRF1 and TRF2 to generate the closed state (van Steensel & de Lange 1997, Smogorzewska *et al,* 2000). TRF2 makes a further contribution to the control of TL by its role in the maintenance of the t-loop (Stansel *et al,* 2001).

Pot1 can also play a role in the maintenance of telomeric DNA content. This occurs as a function of the interaction between Pot1, TRF1 and the single stranded 3' overhang section of the telomere. It is the presence of Pot1 at the 3' overhang which directly inhibits the interaction between the RNA template and DNA primer. Thus in the most simple terms Pot1 acts as a negative regulator of TL by inhibiting telomerase (Loayza & De Lange 2003, Kelleher *et al,* 2005).

1.3.2.5 Epigenetic Regulation

Epigenetic regulation of telomeric heterochromatin signatures by methylation of subtelomeric DNA, in addition to the epigenetic regulation of the expression of telomerase subunits is an important mechanism in the maintenance of TL. Alteration of the methylation status of telomeric chromatin by knockout of the SUV39H1 and SUV39H2 methyltransferases resulted in hypomethylated telomeres which showed an impaired ability to elongate (Garcia-Cao *et al,* 2004). This effect may be linked to alteration in the ability of components of the shelterin complex to regulate telomerase activity (Benetti *et al,* 2007).

Delineating the effect epigenetic modulation of the hTERT gene on TL has proved troubling with both hypo- and hypermethylation of the hTERT promoter and associated histones shown to impact on hTERT expression and TL (Devereux *et al,* 1999, Guilleret & Benhattar 2003, Atkinson *et al,* 2005). The discovery of methylation status contributing to TL control is significant. Methylation can be intrinsically controlled but nutrition may also play a role hence both of these factors could impact on TL (Paul 2011).

1.3.2.6 Alternative Lengthening of Telomeres

Telomerase is responsible for the maintenance of TL in approximately 85-90% of human cancers, a process known as alternative lengthening of telomeres (ALT) is responsible for the remainder. ALT is thought to occur by a homologous recombination mechanism which elongates telomeres independent of telomerase. This confers a number of phenotypic changes in cells that have undergone this process and a number of controlling factors have been identified. However, the reason why some cell types utilise this method of telomere

maintenance as opposed to telomerase dependent mechanisms is not clearly understood (Cesare & Reddel 2010).

Homologous recombination (HR) is thought to represent the most conserved method of ALT. ALT was first reported in mammalian cells by Murnane *et al* (1994) and confirmed by Bryan *et al* (1995) when it was noted that in cells without telomerase, telomeres were highly heterogenous, with rapid changes occurring on longer telomeres (Murnane *et al,* 1994, Bryan *et al,* 1995). The current thoughts are that the telomere replicates itself via the t-loop or copying of a sister chromatid (Cesare & Reddel 2010). Master control of the ALT process resides with the telomere associated proteins, in particular the MRN complex (Zhong *et al,* 2007). One of the key phenotypic changes seen in cells that have undergone ALT is the generation of t-circles. These are thought to form during the resolution of the telomere-loop junction and were first identified by electron microscopy of telomere enriched nuclear extracts (Cesare & Griffith 2004). They may contribute to the ALT process by providing a template for telomeric extension.

1.3.3 Determinants of Telomere Length

1.3.3.1 Age

The relationship between ageing and TL is one that despite being first recognised over twenty years ago still has not been fully delineated. It is clear that TL declines with age as predicted by the end replication problem, but it is not clear whether a cause and effect relationship exists.

Cooke and Smith first linked telomeres to the ageing process with the observation that germ cells had much longer telomeres than adult cells of the non-germ line variety, and that this might be due to the fact somatic cells lacked the recently discovered telomerase (Cooke & Smith 1986). Cooke and Smith thus provided a framework to explain the end replication problem and its relationship with the Hayflick limit. This theory was corroborated with the observation that the TL of fibroblasts in culture reduced with serial passage and that initial TL determined the replicative capacity of similarly cultured fibroblasts (Allsopp *et al,* 1992, Harley *et al,* 1990, Allsopp *et al,* 1995, Vaziri *et al,* 1994). These observations then lead to the theory that TL could act as a mitotic clock in human cells given the dependent relationship of TL with replication (Vaziri *et al,* 1994, Allsopp *et al,* 1995). If the initial observations of Cooke and Smith were true and that telomere

attrition stimulated cells to enter senescence then the consequences of this mitotic clock 'running down' should be entry into senescence. This was confirmed by Allsop & Harley (1995) who demonstrated that a critical TL was responsible for the entry into senescence (Allsopp & Harley 1995). Senescence was by-passed by the introduction of telomerase into human cells which had longer telomeres and reduced markers of senescence (Bodnar *et al,* 1998).

Since these initial *in vitro* observations, efforts have been made to extrapolate them to the organismal level, particularly in human lifespan. Numerous studies have established that as expected human TL falls with increasing chronological age similar to cells *in vitro*. This has been confirmed in various human cell types including peripheral blood leucocytes (Hastie *et al,* 1990, Vaziri *et al,* 1994), colorectal tissue (Nakamura *et al,* 2000), liver (Takubo *et al,* 2002) and pancreas (Ishii *et al,* 2006). TL in post-mitotic tissues such as neurons from the cerebral cortex and cardiac myocytes has been shown to be maintained in relation to chronological age, on a consistent basis (Takubo *et al,* 2002). Results quantifying the annual rate of telomere loss have been variable but the rate of loss usually lies between 20-60bp per year (Takubo *et al,* 2010). However on a population level differences in attrition rates are impacted on by a number of different factors which are potentially modifiable (Shiels *et al,* 2011).

Further refinement of the hypothesis of telomere biology in the ageing process has been possible due to the development of telomerase deficient mouse models. Through deletion of the *TERC* gene mice with phenotypic changes consistent with accelerated ageing are obtained. These include infertility, heart failure, immune deficiencies, tissue atrophy and reduced regenerative capacity. Molecular analysis of these animals revealed critically short telomeres in comparison with control animals, with gross evidence of multiple chromosomal abnormalities indicative of telomere dysfunction (Blasco *et al,* 1997).

In a reverse of the process which produced the original TERC deficient model, late generation $TERC^{-/-}$ mice were crossed with $TERC^{+/-}$ producing offspring in which telomerase was re-activated. These mice had telomeres which were restored to normal length and, moreover, the premature ageing phenotype was rescued (Samper *et al,* 2001). The effect of over-expressing telomerase on lifespan has also been investigated. Initial models of TERT over-expression did have overall longer survival but this was complicated by an increase in sporadic malignancies in some generations (Gonzalez-Suarez *et al,* 2005). In order to circumvent this predicted issue, a mouse model, known as SUPER-M

has been constructed. In addition to over-expression of TERT; p53, p16 and $p19^{ARF}$ tumour suppressors are also constitutively over-expressed. By doing so the incidence of cancer is reduced and therefore dissociated from the ageing process. SUPER-M mice have a delayed onset of age related pathologies, improved overall survival, and longer telomeres in both stem and differentiated cells compared with wild type controls (Tomas-Loba *et al*, 2008). Jaskelioff *et al* (2011) have reversed degenerative phenotypes in multiple organs including testes, spleen and intestines as well as reversing markers of neurodegeneration, in a mouse model in which telomerase activity is induced and TL extended in late generations (Jaskelioff *et al*, 2011).

1.3.3.2 Genetic Determinants

TL is known to be determined in part by intrinsic genetic factors inherited by the individual, and is thought to explain some of the inter-individual variability of TL. Indeed Slagboom *et al* (1994) used 123 human mono- and di-zygotic twin pairs, and calculated 78% heritability in TL (Slagboom *et al*, 1994). Further investigation has revealed that TL is predominantly inherited from the paternal lineage. Paternal age at birth was positively associated with offspring TL, with no relationship demonstrated between maternal age and TL (De Meyer *et al*, 2007). Variability in TL as a result of inherited factors could result from different mechanisms. The finding of paternal age significantly impacting on TL could indicate that TL is not fully re-set after fertilisation and that if paternal TL is short this is inherited by the offspring (De Meyer *et al*, 2007). This is a scenario similar to that seen in animals created by nuclear transfer, in particular when the donor nucleus is harvested from a somatic cell of an older animal (Shiels *et al*, 1999). However, under normal reproductive mechanisms germ cells are the source of parental DNA, which retain the ability to maintain TL using telomerase. This is consistent with the fact that TL at birth is similar between organs of individuals and between sexes, but a wide inter-individual variability remains and further highlights the fact that adult TL is determined in part by intrinsic genetic and in-utero factors (Okuda *et al*, 2002).

1.3.3.3 Sex

Male sex confers a lower mean age of mortality compared with females in a number of different populations. Consistent with this, a number of studies have established that males have shorter overall TL and faster rates of telomere attrition (Njajou *et al*, 2009). However, at birth there is no sex difference in TL indicating that factors which contribute to telomere maintenance, or accelerate attrition, accumulate throughout an individual's lifetime (Okuda

et al, 2002). An obvious mediator of this sex difference later in life is the effect of oestrogen exposure and the menopausal state. Lin *et al* (2011) have recently reported that increased endogenous oestrogen exposure was associated with greater TL suggesting oestrogens may be associated with decelerated cellular ageing (Lin *et al,* 2011). Exogenous hormone replacement of oestrogen and progesterone has also been associated with greater TL in post-menopausal women (Lee *et al,* 2005). In a molecular context oestrogen has been shown to increase telomerase expression by up-regulation of hTERT activity with a concomitant increase in TL (Bayne *et al,* 2011).

1.3.3.4 Oxidative stress

Damage induced by ROS has emerged as a key player in the determination of TL by virtue of accumulating evidence from a number of different experimental systems. The heterogeneity of TL both *in vitro* in cultures cells and *in vivo* is partly explained by the balance of oxidant load and antioxidant defence mechanisms. The intimate relationship between TL and oxidative stress has stimulated some investigators to theorise TL as a biomarker of oxidative damage.

Investigators focusing on the capacity of telomere attrition rates to affect the replicative potential of cells in culture discovered that manipulation of the culture conditions could have a profound effect. Culture of human fibroblasts under mild hyperoxia (oxygen partial pressure 40%) irreversibly blocks proliferation, induces a state indistinct from senescence and is signalled by increased telomere attrition rates with a cut-off of 4Kb (von Zglinicki *et al,* 1995). Treatment of cells cultured under stressful conditions with free radical scavengers abrogates the induced accelerated telomere attrition and can even prolong replicative lifespan (von Zglinicki *et al,* 2000). Whilst cells with an inherent augmented antioxidant capacity such as human foreskin BJ fibroblasts are protected against telomere damaging effects of oxidative stress (Lorenz *et al,* 2001).

A number of diseases in which oxidative stress plays a critical role have been shown to be associated with short telomeres in peripheral blood leucocytes (PBLs) namely, vascular dementia (von Zglinicki *et al,* 2000), atherosclerosis (Brouilette *et al,* 2008) and COPD (Savale *et al,* 2009). Patients with chronic kidney disease (CKD) on dialysis suffer from increased mortality due to an excess of a number of pathologies related to oxidative stress. In these patients this excess risk of mortality is mediated by telomere attrition with a dependant relationship between fetuin-A a circulating inhibitor of calcification and mediator of oxidative stress, and short TL (Carrero *et al,* 2008).

Variation in endogenous antioxidant mechanisms could explain some of the variability in TL at the *in vivo* level. Starr *et al* (2008) correlated a panel of 384 SNPs from 141 oxidative stress genes with TL, with two genes in particular displaying a significant association (Starr *et al,* 2008). Furthermore, increased consumption of antioxidant vitamins has also been confirmed as an independent correlate of longer TL in a female population (Xu *et al,* 2009).

Telomeres exhibit a number of features which makes them particularly susceptible to oxidative damage. The base guanine is particularly sensitive to oxidative damage which is reflected in the level of modified guanine bases particularly 8-oxodG, in senescent cells exposed to oxidative damage (Kawanishi & Oikawa 2004). Oxidative damage induces single strand DNA breaks in telomeric repeats causing interference with the replication fork at telomere ends leading to enlargement of unreplicated ends (von Zglinicki 2002). Moreover, telomeres are less proficient at repair of single-strand breaks than the remainder of the genome, as indicated by the enhanced level of repair in minisateillites compared with telomeres in cells exposed to oxidative damage (Petersen *et al,* 1998). This impairment of damage repair at the telomere may in part be explained by the positioning of TRF2 at the telomere end and has been shown to block the access of DNA repair enzymes to telomeric strand breaks (Richter *et al,* 2007).

Endogenous ROS is mainly derived from mitochondria as an inevitable by-product of oxidative metabolism, with damage occurring due to an imbalance between ROS generation and scavenging pathways (Shiels & Davies 2004, Muller *et al,* 2007). A pro-inflammatory state is a well recognised stimulator of excess ROS production mainly from immune cells such as macrophages and neutrophils. Hence, patients suffering from conditions typified by a chronic inflammatory response have been shown to display evidence of telomere attrition, which is postulated to be as a result of excess ROS generation. Typical conditions include inflammatory bowel disease (Risques *et al,* 2008b), chronic pancreatitis (Hashimoto *et al,* 2008) and type II diabetes mellitus (Sampson *et al,* 2006).

Exogenous or environmental sources of ROS include cigarette smoke. Cigarette smoke contains ROS but can also stimulate endogenous generation by virtue of its pro-inflammatory effects. Several studies have determined a link between cigarette smoke and TL. Indeed, Valdes *et al* (2005) estimated that a 40 pack year smoking history corresponds to 7.4 years of ageing related telomere attrition (Valdes *et al,* 2005).

1.3.4 Telomere Function & Molecular Basis of Dysfunction

As outlined above the maintenance of TL is a stochastic and heterogeneous process whereby a variety of endogenous and external factors co-operate to determine TL. The importance of maintaining an adequate TL is fundamental to the functionality of the telomere, as short telomeres are almost universally dysfunctional.

1.3.4.1 Protection of the Genome & Role in Senescence

Telomeric suppression of DNA damage signalling

The unique nucleo-protein structure of the telomere is essential for its ability to suppress the DDR that would otherwise be initiated if the ends of chromosomes were not protected by the telomere. The most important of these components is the shelterin complex, particularly TRF2 and Pot1. TRF2 has been shown to play a clear role in DDR suppression as exemplified by the fact that deletion of TRF2 leads to accumulation of γH2Ax (a modified histone indicative of DNA damage) and frequent telomere fusions both suggesting de-protection of telomeres (Lazzerini Denchi *et al,* 2006). TRF2 is thought to suppress the initiation of a DDR through direct interaction with ATM the major intermediary in the response to double strand DNA breaks (DSBs). Deletion of TRF2 leads to ATM activation and formation of DNA damage foci at telomeres, which are identical to those formed at sites of DSBs (Karlseder *et al,* 1999, Lazzerini Denchi *et al,* 2006). TRF2 may also suppress the DDR through its ability to create the t-loop configuration of the telomere end thereby preventing the apparatus for detecting DSBs access to the telomere end which would otherwise be recognised as a DNA break (Stansel *et al*, 2001).

A further contribution to the suppression of a DDR is the ability of Pot1 to suppress the ATR pathway. Inhibition of Pot1 triggers the accumulation of DNA damage foci at telomeres, leading to the accumulation of γH2Ax and other factors signifying a damage response (Hockemeyer *et al,* 2005). The ability of Pot1 to repress ATR signalling lies in its interaction with another shelterin component, TPP1. TPP1 recruits Pot1 to the telomere and improves its ability to bind to the G-overhang. Moreover, deletion of TPP1 results in damage foci at telomeres indistinct from those caused by Pot1 deletion (Denchi & de Lange 2007, Hockemeyer *et al*, 2007).

Whether telomere attrition occurs through replicative means as determined by the end replication problem or is accelerated by oxidative damage the end result is indistinct, namely uncapping of the telomere and loss of its key function in suppressing the DDR.

This mainly results from the reduced ability to recruit shelterin complexes in sufficient numbers to maintain TL and, the inability of TRF2 and Pot1 to suppress the DDR as outlined above (Smogorzewska *et al,* 2000, de Lange 2005).

The exact length at which telomeres become uncapped and lose their protective function is a matter for debate and appears to be organism dependent (O'Sullivan & Karlseder 2010). In human fibroblasts in senescence the bulk of telomeres as determined by southern analysis appeared to be around 4kb (Karlseder *et al,* 2002). However, ultra-short telomeres of seven canonical telomeric repeats termed t-stumps have been identified in cancer cells (Xu & Blackburn 2007), whereas in telomerase negative cells repeats of 12.8bp have been noted (Capper *et al,* 2007). It has also been shown that it is the shortest telomere and not average TL that is the main stimulus for senescence (Abdallah *et al,* 2009).

Uncapped telomeres stimulate senescence via initiation of a DNA damage response

The molecular response of the cell to critical telomere attrition and uncapping is a complex interplay between DDR effectors many of which play a dual role in the maintenance of TL. In basic terms, when DDR signalling cascades are initiated in response to telomere attrition cell cycle checkpoints are elicited resulting in either; i) removal of the cell at the G1/S transition by either induction of senescence or apoptosis, or ii) sensing and repair of the damage. Some of the key pathways involved in this process are described below.

Loss of telomeric sequences including bound shelterin complexes results in the activation of the ATM pathway. The exposed DNA is sensed as a DSB by the MRN complex (Table 1.2) and activates ATM, which has been de-repressed by the loss of TRF2 from the telomere complex. A series of reactions then ensues which culminates in the activation of p21 via p53 phosphorylation (Figure 1.7). p21 inhibits cell cycle progression by inactivating pRB and is prerequisite for the induction and maintenance of senescence (d'Adda di Fagagna *et al,* 2003, Herbig *et al,* 2004). As opposed to senescence and depending on the cell type, apoptosis may be triggered in a p53 dependent manner (Karlseder *et al,* 1999). Alternatively, if telomeric damage results in exposure of single stranded DNA then the ATR response pathway is invoked by the de-repression of ATR by Pot1 (Denchi & de Lange 2007) (Figure 1.7). The DDR is actively maintained in senescent cells to prevent future re-entry into the cell cycle, thus telomere initiated senescence reflects a direct DNA damage response checkpoint reliant on dysfunctional telomeres (d'Adda di Fagagna *et al,* 2003).

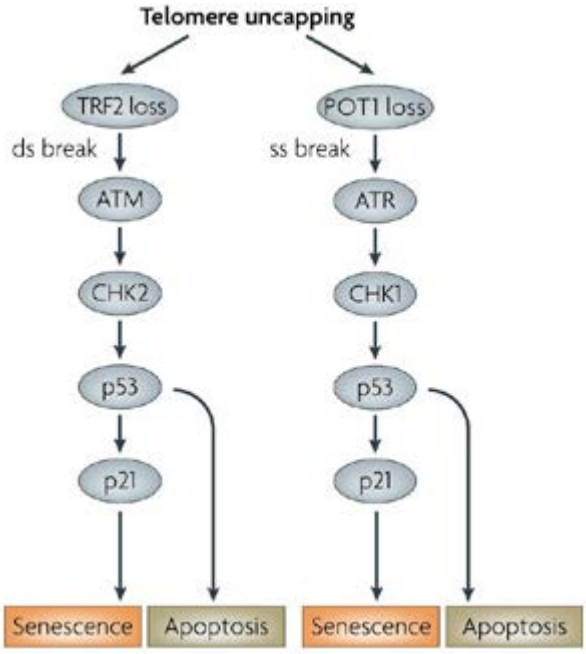

Figure 1.7: Schematic representation of the response elicited by telomere uncapping, modified from Deng *et al* 2008.

Similar to p21, $p16^{INK4A}$ (*CDKN2A*) is a cyclin dependent kinase inhibitor which prevents pRB phosphorylation, maintaining it in an activated state and preventing cell proliferation by inhibition of E2F (a growth factor which increases the transcription of a plethora of genes required for progress through the cell cycle). Both telomeric and non-telomeric DNA damage can engage the $p16^{INK4A}$ pathway but do so with slower kinetics to that of the p53/p21 pathway, and in this setting is seen as secondary to the p53/p21 pathway (Jacobs & de Lange 2005). Cells that senesce solely due to p53/p21 activation can resume growth after inactivation of p53 whereas those fully engaging the $p16^{INK4A}$ /pRB pathway cannot, even after inactivation of p53, $p16^{INK4A}$ and pRB (Beausejour *et al*, 2003). Clearly this finding is relevant in the presence of inactivating mutations of p53 when senescence checkpoints can be bypassed leading to uncontrolled proliferation.

Uncapped telomeres result in chromosomal instability

Short dysfunctional telomeres not only initiate a DDR and senescence, but also allow deleterious repair reactions to occur, namely NHEJ and HR. NHEJ in particular results in 'end to end' chromosome fusions, which are dicentric and therefore cannot be segregated in mitosis. The consequence of this is bridge breakage fusion events, translocations, karyotypic instability and aneuploidy (Riboni *et al*, 1997) (Figure 1.8).

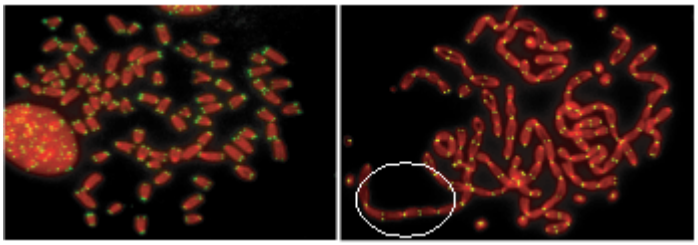

Figure 1.8: Slide to the left indicates normal chromosomal arrangement. Slide to the right shows karyotype after NHEJ induced by dysfunctional telomeres (indicated by circle).

1.3.4.2 DNA Replication

Facilitation of DNA replication is an integral function of the telomere, which is performed by permanent and dynamic telomere associated proteins. As mentioned previously complete telomere (and hence complete chromosome) replication is only possible through the unique action of telomerase. However, even in the absence of telomerase (when telomeric sequences will be lost due to the end replication problem) a functional relationship is required between DNA replication machinery and the telomere associated proteins. For example, telomere replication requires the RecQ helicase WRN in a TRF2 dependent relationship (Crabbe *et al,* 2004). Verdun *et al* (2005) showed that telomeres recruit elements of the MRN complex and ATM in the G2 phase of the cell cycle. This led the authors to conclude that a localised DDR at telomeres is essential for recruiting the processing machinery that promotes formation of the chromosome end complex (Verdun *et al,* 2005). This relationship highlights the intricate nature of the interplay between proteins which have a seemingly paradoxical relationship by promoting efficient telomeric replication, but also sensing and signalling telomere damage and initiating cell cycle checkpoints.

1.3.4.3 Telomeres are required for Efficient Meiosis

Attachment of telomeres to the nuclear envelope in meiosis is required to promote accurate chromosome pairing, by reducing the freedom of movement of sequences near telomeres and limiting chromosomal attachment sites (Gilson *et al,* 1993). This gives rise to the ‘bouquet’ arrangement of chromosomes in meiotic prophase and is dependent on interaction between the telomere and the nuclear membrane protein SUN1 (Ding *et al,* 2007). The consequences of inadequate chromosomal pairing and bouquet formation is

apparent in SUN1 knockout mice where massive apoptotic events are induced leading to the abolition of gametogenesis (Ding *et al*, 2007).

1.3.5 Epidemiological Aspects of Telomere Research

Maintenance of TL is clearly a complex process and determining the exact relationship between telomere dynamics with both ageing and cancer proving similarly difficult. Some of the reasons for this are methodological and explained by variation in the means used to determine TL in index studies. These methodological differences may preclude TL use as a true biomarker of ageing, as was first proposed by the discovery of TL attrition with increasing chronological age.

1.3.5.1 Methods of Telomere Length Measurement

The main methods used to determine TL are southern blot analysis, quantitative fluorescence in-situ hybridisation (q-FISH) and quantitative-PCR (q-PCR). Each of these techniques harbours a number of advantages and disadvantages, which could potentially determine the significance of results gained through their use. The techniques and related comments are outlined in the table below (Table 1.4).

Table 1.4: Table displaying summary of the main mechanistic details of the commonly used techniques to determine telomere length including the advantages and disadvantages of each (Aubert *et al*, 2011).

Technique	Method	Advantages	Disadvantages
Southern Blotting	• Restriction enzyme cut DNA, separated by electrophoresis and transferred to membrane. • Hybridisation with specific telomere probe. • 'Smear' signal converted to Kb TL by various algorithms.	• Thought to be most accurate method and 'gold standard'. • Reproducible	• Time consuming • Large quantity of DNA required. • Relative insensitivity for very short telomeres.
Q-FISH	• Uses image cytometry and metaphase spreads. • Uses high affinity peptide nucleic acid probes which hybridise to denatured telomere DNA repeats. • Fluorescent signal detected and measured relative to known telomere length.	• Allows simultaneous karyotyping. • Microscopically identified cells can be selected for analysis. • Single telomere analysis possible.	• Difficult to achieve semi- quantification of TL due to hybridisation factors. • Unable to measure TL in terminally senescent or highly aberrant cells.
Q-PCR	• Based on the method fist reported by Cawthon (2002). • Uses primers which anneal to C- and G-rich strands of the telomere but have mismatches across their length leading to less primer dimer formation. • Amplification measured quantitatively and compared with that of a single copy gene (S). • Generates a ratio between telomere (T) and single copy amplification termed T/S ratio. • Recently modified as monochrome multiplex technique (Cawthon 2009).	• High throughput possible as less labour intensive. • Large numbers of samples analysed in short space of time. • If performed with required precision and controls accurate comparisons with southern blots achievable.	• Concerns over reproducibility. • Small errors can result in significant alterations in T/S (improved with introduction of monochrome technique).

The convenience of the q-PCR methodology to determine TL has lead to its widespread use as the method of choice in the majority of experimental, clinical and epidemiological studies. However, as outlined in Table 1.4 this methodology is fraught with technical considerations, which may confound results and lead to misleading conclusions. The main problem associated with the technique is the variability in data leading to equivocal results and poor correlation between data derived from southern blotting, the so-called 'gold standard' method (Aviv 2004, Aviv 2008, Aviv 2009). These differences have stimulated leaders in the field to call for uniformity of methodology in an attempt to reduce variability

and prevent confounding factors skewing results. Potential ways in which variability could be reduced includes; double blind analysis of samples, standardisation of DNA preparation, inclusion of suitable controls and introduction of a worldwide laboratory standard operating procedure (Shiels 2010, Martin-Ruiz *et al*, 2011). Despite potential issues with the technique q-PCR is a useful tool for researchers in the field of telomere biology and its relation to ageing and disease. However, it is imperative that conclusions drawn from studies using the technique are done so in the context of the potential limitations.

1.3.5.2 Telomere length as a Biomarker of Ageing

The initial observations of a decline in TL with chronological age lead to the introduction of the concept of TL as a biomarker of aging. However, as it became clear that telomere dynamics were subject to manipulation by numerous intrinsic and extrinsic forces the emphasis was shifted more towards using TL as a marker of cellular bio-ageing.

Baker and Sprott (1988) outlined the criteria considered prerequisite for any marker to be classified as a biomarker of ageing. They defined such a marker as:

"a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease better predict functional capability at some late age, than will chronological age" (Baker & Sprott 1988).

Although the change in TL with chronological age is incontrovertible the use of TL as a marker of ageing has recently been questioned. Mather *et al* (2011) have recently reviewed the literature pertinent to the utilisation of TL as a biomarker of ageing and suggest that whilst there is clear evidence that telomeres are involved in ageing and diseases of premature ageing, the data supporting TL as a biomarker of ageing is inconclusive (Mather *et al*, 2011). There are a number of potential reasons highlighted, the high degree of inter-individual variability at similar chronological ages, the lack of definitive association between TL and functional capacity, and the methodological reasons outlined above. To be a useful biomarker TL should predict lifespan better than chronological age. Studies investigating this specific issue are inconclusive. Cawthon *et al* (2003) first reported an overall increase in mortality with reduction in TL. This excess mortality was attributed to

higher mortality from cardiovascular and infectious disease (Cawthon *et al*, 2003). A recent report has also confirmed a weak association between short TL and mortality in an elderly population (Fitzpatrick *et al*, 2011). However, several studies have failed to conclusively demonstrate that short TL predicts mortality (Bischoff *et al*, 2005, Harris *et al*, 2006).

1.3.6 Telomere Dysfunction & Cancer

As outlined above, telomeres not only act as a cellular marker of replicative potential but they also function to maintain genomic integrity and facilitate accurate chromosomal replication. Integral to the ability to maintain genomic integrity is the capacity to induce cell cycle checkpoints, allowing repair of sensed damage, or if the damage is deemed irreparable removal of the cell from the proliferating population by apoptosis or senescence. It is therefore no surprise that acceleration of telomere attrition, or aberration of any of the elements maintaining optimal telomere dynamics leads to the development of chromosomal abnormalities which are not just typical of transformed cells, but essential for the initiation of neoplasia. The body of evidence implicating altered telomere biology in the cancer process is increasing rapidly. Some of the postulated mechanisms and *in vivo* studies providing the rationale for this indelible relationship will be discussed below.

1.3.6.1 Mechanisms of Tumourigenesis

Dysfunctional Telomeres & p53

In an attempt to characterise the relationship between telomere attrition, p53 and malignant transformation a number of mouse models have been developed. These have used the aforementioned *TERC*$^{-/-}$ mouse crossed with mouse models designed to reflect aberrancy of different components of the telomere complex and DDR mechanism. In the context of the relationship between human cancer and ageing perhaps the most important observation results from the *TERC*$^{-/-}$,*Trp53*$^{-/-}$ (p53 null) mouse. In these animals progressive telomere attrition corresponded with increased tumour incidence and decreased survival. Moreover, late generation mice succumbed to a range of tumours strikingly similar to those seen in elderly humans, namely breast, skin and intestinal. Cytogenetic analysis also revealed

chromosomal aberrations typical of these tumour types (Artandi *et al,* 2000, O'Hagan *et al,* 2002). This model emphasises the importance of mutated p53 in the initiation of tumourigenesis in the setting of critically short telomeres (Chin *et al,* 1999).

Models of specific tumour types have also yielded valuable information. The *APC*^{min} mouse model has been designed to develop benign micro and macroadenomas throughout the gastrointestinal tract. When crossed with the *TERC*$^{-/-}$ mouse, early generation offspring develop microadenomas, which progress onto more aggressive and detrimental macroadenomas. However, in later generation animals which have short and dysfunctional telomeres only microadenomas develop (Rudolph *et al,* 2001). This finding of protection against cancer in later generation in animals with short telomeres is observed in a *CDKN2A*$^{-/-}$ mouse deficient in p16^{INK4A} and ARF who develop increased incidence of lymphoma and sarcoma (Greenberg *et al,* 1999). These findings suggest that in the presence of competent tumour suppressive pathways in particular p53, short telomeres elicit a protective function against neoplasia, presumably through initiation of senescence.

Telomere dysfunction drives chromosomal instability

Human tumours particularly of an epithelial origin display large numbers of focal amplifications and deletions. These copy number alterations are thought to drive the process of carcinogenesis by causing activation of proto-oncogenes or inactivation of tumour suppressor genes (Pinkel & Albertson 2005). Spectral karyotype analysis of cancer cells from tumours in mouse models driven by telomere dysfunction reveal similar copy number alterations. These show chromosome end fusions and numerous non-reciprocal translocations leading to aberrant copy number. Furthermore, these have been shown to be a major driver of amplication/deletion events at genes relevant to the initiation and maintenance of neoplasia (O'Hagan *et al,* 2002). These hallmarks are evident not only in the *TERC*$^{-/-}$ mouse model of telomere attrition, but also in the TPP1$^{acd/acd}$ mouse which models telomere uncapping by disrupting the shelterin complex. These mice display increased epithelial cancers particularly of the skin, in a p53 deficient setting with hallmark copy number aberrations (Else *et al,* 2009).

1.4.6.2 Senescence & Cancer

The irreversible growth arrest initiated by senescence makes it an ideal candidate for a role in the prevention of neoplastic transformation and tumour suppression. Indeed senescent

cells have been shown in abundance in a variety of pre-malignant lesions. For example, pre-malignant lesions from the lungs of an oncogenic k-Ras mouse model contained abundant senescent cells, when compared with invasive lung adenocarcinomas (Collado *et al,* 2005). These findings have been corroborated in a mouse model of prostate cancer lacking the tumour suppressor PTEN and importantly, from benign lesions of human skin carrying oncogenic mutant BRAF (Michaloglou *et al,* 2005, Chen *et al,* 2005b). These findings implicate senescence as a barrier of transformation from benign non-invasive lesions to malignancy.

Both oncogene induced and replicative senescence have been shown to exert a tumour suppressive role. Analysis of benign naevi showed high levels of senescence but no discernible reduction in TL, indicating that oncogene-induced senescence rather than replicative senescence may confer the limitation of proliferation in these lesions (Michaloglou *et al,* 2005). Meanwhile, complex inter-generational crossing of the telomerase knockout mouse with the *Eμ-Myc* transgenic model of Burkitts lymphoma revealed hallmarks of chromosomal instability due to telomere dysfunction in tumour cells of late generation animals. Furthermore, over-expression of the anti-apoptotic gene *Bcl2* in cells from late generation crossed animals with subsequent re-implantation resulted in lymph node tumours which were small and proved positive for markers of senescence such as $p16^{INK4A}$. These results suggest that dysfunctional telomeres can induce senescence in the absence of apoptosis to exert a tumour suppressive effect (Feldser & Greider 2007). Similarly, telomerase null mice in which the pro-apoptotic effect of p53 was ameliorated but p53-dependant senescence pathways maintained, displayed near universal reduction in tumour formation (Cosme-Blanco *et al,* 2007).

Whilst the studies mentioned above provide evidence of senescence acting as a barrier to malignant transformation, some of the characteristics of senescent cells confer tumour promoting properties. The development of the secretory associated senescence phenotype (SASP) can alter the behaviour of neighbouring cells via the secretion of mostly pro-inflammatory cytokines and growth factors known to stimulate various characteristics of neoplasia (Rodier & Campisi 2011). Human cells subjected to DNA damage adopt the senescence phenotype and secrete IL-6 in a manner dependent on the DDR proteins ATM, NBS1 and Chk2. Furthermore, IL-6 promoted human cancer cell invasiveness in a dependent relationship with ATM (Rodier *et al,* 2009) and stimulated both pre-malignant and weakly malignant cells to penetrate the basement membrane (Coppe *et al,* 2008). In

addition, vascular endothelial growth factor (VEGF) released by senescent cells can stimulate endothelial cell migration and invasion, both key steps in the process of neoangiogenesis (Coppe *et al,* 2006). Senescent cells also harbour the ability to secrete matrix metalloproteinases (MMPs) particularly MMP-1, -3 and -10. These proteases contribute to the tissue remodelling required for tumour cell invasion (Davalos *et al,* 2010). It is therefore plausible that senescent cells could play a role in the progression and development of neoplasia via paracrine signalling to neighbouring cells (Campisi & d'Adda di Fagagna 2007). In support of this paradigm Krtolica *et al* (2001) reported that co-injection of senescent cells into xenograft models of malignancy stimulated pre-malignant lesions to become malignant and accelerated the rate of tumour formation when co-injected with malignant cells (Krtolica *et al,* 2001). The seemingly dichotomous relationship between senescence and cancer can be explained in evolutionary terms as a further example of antagonistic pleiotropy. Whereby tumour suppression by senescence is a protective mechanism in younger individuals but, as an individual ages and deleterious mutations accumulate its effect becomes pro-tumourigenic (Coppe *et al,* 2010).

1.3.6.3 Telomere Length and Human Cancer Risk

Numerous studies have now been published with the aim of delineating the relationship between human TL and cancer risk. The methodologies used to measure TL, the source of telomeric DNA and the cancer populations studied have been diverse. On the whole the results have indicated that short TL is associated with increased cancer risk. However, some studies have produced conflicting results with both short and long TL associated with increased cancer risk in some histological subtypes. These differences are difficult to rationalise however inherent differences in the methodologies used and the natural history of the cancer type under investigation could explain some of the heterogeneity.

Leucocyte Telomere Length & Cancer Risk

The rationale behind using peripheral blood leucocytes (PBLs) as a source of telomeric DNA is one borne of convenience. Evidently it is easier to obtain a patient blood sample than a sample of tumour cells, particularly of solid tumours. The use of PBLs may also give a more general indication of telomere dynamics as opposed to the tumour level where the reactivation of telomerase will stabilise TL to enable enhanced proliferation. TL in

different cell compartments have been shown to be comparable, indicating PBL TL could serve as a surrogate parameter of the tissue under investigation (Friedrich *et al,* 2000).

Over the last ten years the number of studies documenting an association between PBL TL and cancer risk has steadily increased. The first study to investigate any potential link was published by Wu *et al* (2003) using a retrospective case-control study design and southern blot analysis to determine TL. The authors reported a significant association between short TL and bladder, lung, head and neck and renal cell cancer (Wu *et al,* 2003). Since these initial findings numerous cancer types have been investigated producing similar results. Short TL has been associated with an increased risk of breast (Shen *et al,* 2009), bladder (McGrath *et al,* 2007), ovarian (Mirabello *et al,* 2010), gastric (Hou *et al,* 2009), colorectal (Pooley *et al,* 2010) and oesophageal cancer (Xing *et al,* 2009). Conversely some studies have reported an equivocal risk of colorectal (Zee *et al,* 2009, Lee *et al,* 2010), prostate (Mirabello *et al,* 2009) and breast (Kim *et al,* 2011) cancer with short TL. Whilst, some studies have even reported an association between long telomeres and an increased risk of colorectal (Jones *et al,* 2011), breast cancer (Svenson *et al,* 2008) and clear cell renal cancer (Svenson *et al,* 2009). These conflicting results clearly indicate a degree of heterogeneity between studies. Such sources could relate to the method used to determine TL or the study population under investigation. The natural history of the cancer type under investigation could also potentially confound results, as could the retrospective design of most of the studies. Willeit *et al* (2010) therefore employed a prospective longitudinal study design to investigate cancer risk in 787 healthy participants followed-up over a period of 10 years. They reported that both cancer risk and mortality from cancer was increased in patients in the shortest telomere group when compared with the longest (Willeit *et al,* 2010).

Overall cancer risk has been shown to be affected by genetic variation in expression of telomere-associated proteins. Four SNPs in the TERT and Pot1 genes were associated with increased breast cancer risk (Shen *et al,* 2010). Similarly variation in the TERT and TRF1 gene was associated with skin cancer risk (Nan *et al,* 2011), with TERT and TNKS variability associated with susceptibility to lung cancer (Choi *et al,* 2009). Whilst it is clear genetic variability influences cancer risk the direct relationship with TL can only be speculated on as concomitant measurement of TL was not performed in these studies.

Tissue telomere length & Cancer Risk

Measurement of cancer tissue TL has the added advantage over PBL TL that direct correlation of TL from pathological specimens with outcome measurements could provide a novel addition to routine biomarkers of risk stratification. Several studies in a number of tumour subtypes have attempted to provide prognostic information (Bisoffi *et al,* 2006). Short TL has been shown to be associated with adverse pathological features, metastasis and poor prognosis in breast cancer (Griffith *et al,* 1999b, Fordyce *et al,* 2006). Similarly TL in prostate cancer sample specimens predicts time to recurrence independent of age, Gleason score and pelvic node involvement (Fordyce *et al,* 2005). However, this correlation between TL and adverse outcomes was not replicated in colorectal cancer where longer TL was associated with poor outcome and more advanced disease. Although, as in the previous studies mentioned in different tumour subtypes TL in cancer specimens was significantly shorter than adjacent normal tissue (Gertler *et al,* 2004, Garcia-Aranda *et al,* 2006). These differences may result from the behavioural characteristics of colorectal cancers relating to the natural history of the progression through the adenoma-carcinoma sequence of events leading to cancer development in this tissue (Kim *et al,* 2002, Plentz *et al,* 2003).

Clearly a number of aspects of telomeric function are altered in the development and progression of oncogenesis. In order to provide a molecular context for this a number of pathways which intricately link ageing and cancer were considered. For example, the ability of a cell to optimally respond to stressful stimuli is dependent on its ability to sense the damage and respond accordingly, in the face of the metabolic and oxidative conditions at that time. It has been postulated that one way in which this critical cellular response can be rationalised is via the concept of the MTR trinity (Mitochondrion Telomere and Ribosome biogenesis). Through this triad critical DNA damage is sensed by the telomere nucleo-protein complex which also plays a role in effector mechanisms such as senescence. Energy production and apoptosis are facilitated by the mitochondria and energy utilisation, as well as the requisite protein synthetic pathways are controlled via rDNA (Shiels & Davies 2004). A potential linking factor of these individual elements of the MTR are a group of nutrient sensing proteins termed the sirtuins. These proteins control a number of critical cellular functions fundamental to both the ageing and cancer with aberrancy of their expression linked to both processes. A full description of this interesting group of proteins will now follow.

1.4 Sirtuins

1.4.1 Silent Information Regulator 2 (Sir2)

The term Silent information regulator 2 (Sir2) was coined to describe the product of a unique class of genes first discovered in the budding yeast *Saccharomyces cerevisiae*, which function to silence gene transcription at the silent mating type loci. This contributes to a number of different mechanisms which control gene expression at the level of transcription at specific chromosomal domains. Transcriptional silencing involves induction of a specialised chromatin structure that physically impedes transcription as well as a number of other processes, including epigenetic modification by methylation (Guarente 1999, Gartenberg 2000). Following intensive investigation it has become clear that Sir2 is part of a family of genes phylogenetically conserved from bacteria to man more broadly termed the sirtuins. However, it was not until Sir2 was implicated in the control of yeast longevity and latterly the ageing process in general that interest increased, giving rise to the prospect that manipulation of these genes or their products could affect human longevity or even reverse the ageing process (Kaeberlein *et al*, 2007, Kaeberlein 2008).

1.4.2 MAMMALIAN SIRTUINS

The field of ageing research was fundamentally changed with the discovery that Sir2 and its homologues not only controlled key cellular processes but also longevity in model organisms (Kaeberlein *et al*, 1999). Work was therefore initiated to determine whether the same effects were evident in higher organisms including humans. To date seven homologues of Sir2 termed sirtuin 1-7 have been discovered in mammals, these have varying sub-cellular localisations and functions compatible with critical regulators of cellular function. Some of the key aspects of the molecular biology and physiology of the sirtuins are discussed below.

1.4.2.1 Homology with Sir2

Genetic Homology

In mammals there are seven orthologues of the Sir2 gene termed sirtuin (SIRT) 1-7. SIRT1 shares the closest homology with the yeast Sir2, whilst SIRT4 and SIRT5 resemble prokaryotic sirtuin sequences. SIRT6 and SIRT7 more closely resemble each other than the Sir2 gene (Frye 1999, Frye 2000). Analysis of over sixty conserved sirtuin sequences from a varying range of organisms has identified five groups into which the sirtuins can be classified, termed class I-IV and U. Yeast Sir2, SIRT1, SIRT2 and SIRT3 lie within class I. SIRT4 and SIRT5 are class II and class III sirtuins respectively with class IV containing mammalian SIRT6 and SIRT7. Neither class I nor class IV are found in prokaryotes. Class U contains sirtuins from some gram positive bacteria and *Thermotoga maritima* in which the sequence motifs are intermediate between class I to IV (Frye 2000).

Structural & Enzymatic Homology

The basic structure of the mammalian sirtuins resembles that described previously in yeast, consisting of a large domain of a classical open α/β, Rossman fold structure and a smaller domain. The main enzymatic function of yeast Sir2 is as a histone deacetylase requiring NAD^+ as a dependant cofactor, which is conserved by the mammalian sirtuins (Imai *et al,* 2000). The NAD^+ binding site was discovered to be situated within a pocket in which the large domain forms the floor and the small domain the ceiling. The conserved domain contains a motif of Cys-X-X-Cys-(X)15-20-Cys-X-X-Cys which is thought to bind a Zn^+ ion (Min *et al,* 2001).

The utilisation of NAD^+ as a cofactor for the deacteylase activity of the mammalian sirtuins is maintained by SIRT1, SIRT2, SIRT3, SIRT5 and SIRT6 (Schwer *et al,* 2002, Michishita *et al,* 2005, Michishita *et al,* 2009, Nakagawa *et al,* 2009). In addition SIRT4 and SIRT6 have been shown to possess robust ADP-ribosyl-transferase activity, a reaction which also requires NAD^+ as a fundamental cofactor (Liszt *et al,* 2005, Haigis *et al,* 2006, Michishita *et al,* 2009). The control of the deacetylase activity is dependent on the NAD^+/NADH ratio within the cell. This requirement of NAD^+ as a cofactor for the catalytic activity of Sir2 and the sirtuins in general raises the intriguing possibility of altering key cellular processes implicated in organismal ageing through varying availability of metabolic intermediates, potentially by dietary manipulation (Guarente 2000).

1.4.2.2 Sirtuin Sub-Cellular Localisation

The most extensive study investigating the cellular localisation of the sirtuins was undertaken by Michishita and colleagues (2005). SIRT1, SIRT6 and SIRT7 were found mainly in the nucleus, however there was some sub-nuclear variation in that SIRT1 appeared to be excluded from the nucleoli and SIRT6 was found overlapped with heterochromatic regions (Michishita *et al,* 2005). SIRT3 was already known to reside in mitochondria (Schwer *et al*, 2002).This was confirmed and expanded upon by data demonstrating that SIRT4 and SIRT5 co-localised with a mitochondrial marker confirming SIRT3, SIRT4 and SIRT5 as mitochondrial sirtuins (Michishita *et al,* 2005). Consistent with its role as a tubulin deacteylase SIRT2 is predominantly found in the cytoplasm, however during mitosis resides in the nucleus (North *et al,* 2003).

Some of the major features of the mammalian sirtuins including genetics, activity and localisation are summarised in Table 1.5.

Table 1.5: Table displaying some of the common characteristics of SIRT1-7.

Sirtuin	Class	Chromososme	Cellular Localisation	Enzymatic Function	Targets	Cellular Function
SIRT1	I	10q21.3	Nucleus, cytoplasm	Deacetylase	H1, H3, H4 p53, NF-κB, ku70, FOXO, PGC-1α,	Cell survival, glucose metabolism, inflammation, neurodegeneration
SIRT2	I	19q13.2	Cytoplasm	Deacetylase	H4, α-tubulin	Cell cycle control
SIRT3	I	11p15.5	Mitochondria	Deacetylase	Acetyl CoA synthetase 2, long chain acyl CoA dehydrogenase	Thermogenesis, metabolic response
SIRT4	II	12q24.23	Mitochondria	ADP-ribosyl transferase	Glutamate dehydrogenase	Insulin secretion/metabolic control
SIRT5	III	6p23	Mitochondria	Deacetylase	Carbamoyl phosphate synthetase-1	Control of urea cycle
SIRT6	IV	19p13.3	Nucleus	Deacetylase ADP-ribosyl transferase	Telomere histone H3, HIF-1α, DNA-dependent protein kinase	Maintenance of genomic integrity, DNA repair, insulin secretion
SIRT7	IV	17q25	Nucleus	Deacetylase	RNA polymerase I	rDNA transcription, cell proliferation

This variability in the sub-cellular localisation and targets with which they interact is reflected in the diverse fundamental cellular processes under the control of the sirtuins. The sirtuins, in particular SIRT1 have been demonstrated to regulate not only histones but a multitude of other non-histone proteins which control a number of key processes such as cell survival, stress resistance, metabolism and protection of genomic integrity. Clearly, these processes need to be controlled with fine precision as they are intricately linked not only to normal and premature ageing but also to some of the deleterious pathological outcomes associated with ageing, in particular neoplasia. The key features and functions of each of the sirtuins will now be discussed.

1.4.3 SIRTUIN 1

Cloning studies have determined that the human *Sirt1* gene spans 33,660 bp over one genomic locus. FISH analysis defined the position of the *Sirt1* gene to chromosome 10q21.3. Whilst, phylogenetic analysis revealed the human *Sirt1* gene shared 56% pair wise homology with yeast Sir2, the highest of any the human sirtuins (Voelter-Mahlknecht & Mahlknecht 2006). Consistent with its place in the group of histone deacetylases SIRT1 exerts a controlling influence on chromatin and hence epigenetic phenomena. SIRT1 displays strong deacetylase activity towards lysine 26 on histone H1 (H1K26), H4K16 and H3K9. RNA interference to knockdown SIRT1 expression reveals hypoacetylation at these and other key sites suggesting a key involvement of SIRT1 in heterochromatin regulation (Vaquero *et al*, 2004). SIRT1 can further modify chromatin via its ability to deacetylase and regulate key methyltransferases, namely SUV39H1. The acetylation of H4K16 is thought to be particularly important for optimal chromatin function and is evolutionarily conserved (Vaquero *et al*, 2004, Vaquero *et al*, 2007).

1.4.3.1 SIRT1 Controls Key Homeostatic Processes

Cell Survival & Response to Stress

The main feature of SIRT1 which distinguishes it from the remainder of the sirtuins and its yeast homolog Sir2 is its ability to deacetylate a multitude of non-histone targets including p53, FOXO, NF-κB and Ku70 (Yang *et al*, 2005, Jeong *et al*, 2007, Ghosh *et al*, 2007, Yi & Luo 2010) (Figure 1.9). This confers SIRT1 with the capacity to regulate a number of key cellular processes and adaptive mechanisms, allowing the tailoring of stress response to the level of any external insult such as CR or genotoxic stress, and dictating cellular fate by modulating apoptotic pathways and intermediaries (Milner 2009).

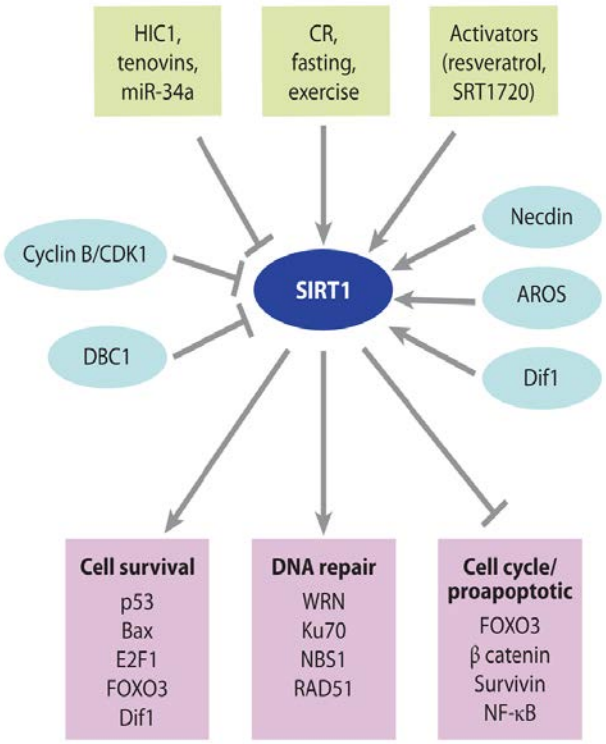

Figure 1.9: Some of the processes and downstream targets through which SIRT1 acts to regulate cell survival and response to stress. Taken from (Haigis & Sinclair 2010).

SIRT1 Interaction with p53- Apoptosis & Senescence

Two separate studies identified p53 to be the first SIRT1 non-histone target. These studies demonstrated that p53 was a SIRT1 target and that the p53 pro-apoptotic response to stress was repressed by SIRT1 induced deacetylation of p53. Whilst expression of dominant negative SIRT1 potentiated the p53 controlled cellular stress response (Luo *et al*, 2001, Vaziri *et al*, 2001).

SIRT1 mediates an effect over the two known pathways of p53 induced apoptosis. Transcription independent apoptosis is modulated via SIRT1 control over p53 sub-cellular localisation. This was demonstrated by Han et al (2008) in mouse embryonic stem cells (mES) cells where SIRT1 blocked nuclear translocation of cytoplasmic p53 in response to endogenous ROS (Han *et al*, 2008a). Transcription dependant apoptosis is a finely balanced process involving p53 mediated upregulation of pro-apoptotic genes such as Bax, PUMA and NOXA (Yu & Zhang 2008). SIRT1 has the ability to influence this pro-apoptotic pathway by deacetylating members of this intricately controlled process. For

example, deacetylation of Ku70 alters the Ku70/Bax interaction resulting in an altered apoptotic response (Cohen *et al,* 2004a).

The effect of SIRT1 on p53 mediated senescence is complex with the results of studies seemingly at odds. SIRT1 represses p53 activation by deacetylation thereby antagonising premature senescence in MEFs (Langley *et al,* 2002). However, in contrast with these findings SIRT1 deficient MEFs displayed an enhanced resistance to replicative senescence under conditions of chronic sublethal genotoxic stress induced by H_2O_2, an effect which is mediated by $p19^{ARF}$ and its ability to positively regulate p53 (Chua *et al,* 2005). Thus SIRT1 has two distinct mechanisms of influencing p53 function, by direct deacetylation and through regulation of $p19^{ARF}$. These have opposite affects on net p53, thus highlighting the complexity of the role SIRT1 plays in the control of senescence. It is likely that the overall effect is dependent on the trigger of cellular stress and the apoptotic signalling mechanism induced.

SIRT1 and other Non—Histone Targets

FOXO transcription factors have been shown to up-regulate the transcription of genes controlling apoptosis, cell cycle arrest and differentiation. Integrated responses to stressful stimuli are achieved by a number of mechanisms including modification of acetylation status by SIRT1. SIRT1 has been shown to deacetylate FOXO1, FOXO3 and FOXO4 but the overall effect of deacetylation is complex (Brunet *et al,* 2004, Daitoku *et al,* 2004, Motta *et al,* 2004, van der Horst *et al,* 2004). Deacetylation can result in activation of FOXO1 and FOXO4 with subsequent upregulation of target genes involves in stress resistance, or inhibition of FOXO3 dependant apoptosis and promotion of DNA repair (Brunet *et al,* 2004, Daitoku *et al,* 2004, Kobayashi *et al,* 2005, Yang *et al,* 2005). The overall effect appears to be one of shifting FOXO-dependant responses towards cell survival by stress resistance and inhibition of apoptosis, leading to cell cycle arrest.

NF-κB controls key processes involved in cell cycle control, cell fate and is paramount for both adaptive and innate immune responses. Once activated it translocates to the nucleus where it enhances transcription by tethering histone acetyl transferases (HATs). SIRT1 physically interacts with the RelA/p65 subunit of NF-κB resulting in its deacetylation and inactivation, thus SIRT1 activity augments TNF-α induced apoptosis (Yeung *et al,* 2004). Thus the effect of the interaction between SIRT1 and NF-κB is to positively regulate apoptosis whereas interaction of SIRT1 with p53, Ku-70 and FOXO results in the

attenuation of apoptosis, promoting cell survival. It is therefore clear that the overall effect of SIRT1 on cell survival is varied and complex. Some of the reported variations can be explained by the response induced by the different types of experimental stimuli utilised. For example induction of the DNA damage response by γ-irradiation stimulates ATM dependant accumulation of p53 and ultimately upregulation of pro-apoptotic gene products whilst TNF-α induces p53-independant apoptosis which is repressed by NF-κB (Yi & Luo 2010). Further variation in response depending on the cell type used is almost certainly inevitable. It stands to reason that aberrancy of expression of SIRT1 or any of the mediators involved in the signalling pathways controlling cell survival leading to altered longevity and cell turnover with the obvious implications for ageing and cancer.

Metabolic Regulation

SIRT1 plays a role in glucose homeostasis by two main mechanisms, regulation of insulin secretion and stimulation of hepatic gluconeogensis. Moynihan *et al* (2005) using β-cell-specific SIRT1-overexpressing mice and Bordone *et al* (2006) using $SIRT1^{-/-}$ mice independently demonstrated that SIRT1 positively regulates glucose stimulated insulin secretion in pancreatic β-cells (Moynihan *et al*, 2005, Bordone *et al*, 2006). Modulation of hepatic gluconeogenesis occurs via SIRT1 mediated activation of PGC-1α. Activation of PGC-1α in the liver stimulates gluconeogenesis and inhibits glycolytic pathways via a nutrient signalling response mediated by pyruvate (Rodgers *et al*, 2005).

In addition to its role in glucose homeostasis SIRT1 can regulate adipogenesis and lipolysis via its interaction with PPARγ. Repression of PPARγ by SIRT1 results in attenuation of adipogenesis and upregulation of lipolysis in response to food withdrawal (Picard *et al*, 2004).

Vascular Biology

There is increasing evidence that SIRT1 plays a key role in vascular function and lipoprotein metabolism, translating to a protective effect against cardiovascular disease. SIRT1 activates transcription of liver X receptors (LXR) target gene encoding the ATP-binding cassette transporter A1. This has the effect of increasing high density lipoprotein synthesis with the concomitant benefits on atherosclerotic plaque formation via altered cholesterol transport (Li *et al*, 2007). In addition, SIRT1 has been shown to regulate

vascular tone by deacetylating and stimulating endothelial nitric oxide synthase (eNOS) thereby increasing nitric oxide (NO) levels (Mattagajasingh *et al*, 2007).

Neuronal Function

SIRT1 has been shown in a number of model systems to play a key role in neuronal survival, in particular axonal degeneration. The wallerian degeneration slow (Wld^S) mouse line exhibits delayed axonal degeneration in response to injury, an effect which is mediated by the increased expression of an enzyme (Nmnat1) required for the *de novo* synthesis and salvage of NAD^+ with SIRT1 as a downstream effector (Araki *et al,* 2004). In a more clinical context SIRT1 has been shown to modulate disease activity in Alzheimer's disease (AD) by ameliorating the damage induced by Aβ peptides through an effect on NF-κB signalling (Albani *et al*, 2010).

Differentiation

Terminal cellular differentiation of a number of different histological subtypes provides another mechanism by which SIRT1 can alter tissue dynamics in response to changes in the metabolic micro-environment. SIRT1 can modulate neuronal differentiation by becoming transiently translocated to the nucleus of neural precursor cells (stem-like cells) and repressing the Notch1/Hes1 signalling pathway. However, the functional role of SIRT1 on neuronal development remains to be fully elucidated (Hisahara *et al*, 2008). Skeletal muscle differentiation and gene expression is also subject to regulation by SIRT1 due to dynamic fluctuations in $[NAD^+]$:[NADH] and overexpression of SIRT1 results in immature myotubes with reduced expression of myosin heavy chain and myogenin. Thus gene expression and muscle differentiation is regulated by metabolic demands of muscle that occur in response to food intake, fasting and exercise (Fulco *et al,* 2003).

1.5.4 SIRTUIN 2

FISH analysis of the human SIRT2 gene localises it to chromosome 19q13.1 (Voelter-Mahlknecht *et al,* 2005). SIRT2 is predominantly located in the cytoplasm and expression profiling reveals high levels in foetal brain, adult brain and kidney (Michishita *et al,* 2005).

The bulk of evidence investigating the cellular function of SIRT2 points to its role as a mediator of the cell cycle via its ability to deacetylate tubulin (North *et al*, 2003).

1.4.4.1 Functions of SIRT2

Cell Cycle Control

Functional studies of human SIRT2 have revealed that its expression is abundantly increased during mitosis and in particular at the G2/M transition of the cell cycle when it is also phosphorylated. Cells overexpressing SIRT2 display a marked prolongation of the mitotic phase of the cell cycle (Dryden *et al*, 2003). Further analysis by North and colleagues (2003) revealed the predominant intra-cellular target of SIRT2 to be α-tubulin (North *et al*, 2003). SIRT2 has also been shown to deacetylate lysine 16 on H4 again specifically during the G2/M transition when SIRT2 can be localised on Chromatin (Vaquero *et al*, 2006). The ability of SIRT2 to control mitotic exit from the cell cycle is dependent on its phosphorylation levels. Dephosphorylation of SIRT2 by the phosphatase CDC14B also has the effect of destabilising the SIRT2 protein promoting mitotic exit (Dryden *et al*, 2003). Phosphorylation of SIRT2 has since been demonstrated to be achieved by the cell cycle regulator cyclin-dependent kinase 1 at serine 368 (North & Verdin 2007b). Failure of SIRT2 to control cell cycle exit and act as a checkpoint could allow abnormal chromosomal condensation and therefore chromosomal aberrancy. SIRT2 has therefore been identified as a potential contributor to the chromosomal abnormalities evident in some tumour types.

Tubulin-Independent targets

Pertinent to its potential role in cell turnover and accurate chromosomal replication SIRT2 has recently been demonstrated to interact with the ubiquitous transcription factor NF-κB. SIRT2 can deacetylate the p65 subunit both *in vitro* and *in vivo* at Lys 310. Furthermore p65 is hyperacetylated in $SIRT2^{(-/-)}$ cells stimulated by TNF-α, leading to the increased expression of NF-κB dependant genes (Rothgiesser *et al*, 2010).

1.4.5 SIRTUIN 3, 4 & 5

Collectively SIRT3, 4 & 5 are termed the mitochondrial sirtuins. Localisation of these sirtuins to the mitochondria stimulated great intrigue in the ageing research community given the importance of these organelles in regulating nutrient utilisation, energy production and in apoptosis. Furthermore, as a by-product of the reactions involved in energy production, mitochondria are the primary site of reactive oxygen species generation in the cell (Huang *et al,* 2010).

1.4.5.1 Sirtuin 3

The human SIRT3 gene is located on the short arm of chromosome 11 at position 15.5. It possesses robust NAD^+ deacetylase activity and is localised to the mitochondrial matrix (Onyango *et al,* 2002, Schwer *et al,* 2002, Michishita *et al,* 2005). Some controversy exists as to whether SIRT3 is truly mitochondrial. Upon translation it translocates from the cytoplasm into mitochondria where it is cleaved and activated by matrix processing peptidase (Scher *et al,* 2007). Other studies, however, have confirmed SIRT3 as a purely mitochondrial deacetylase (Hallows *et al,* 2006).

SIRT3 and Metabolic Regulation

Although SIRT3 targets a wide range of mitochondrial proteins for deacetylation the most widely characterised substrate is acetyl-CoA synthetase 2 (AceCS2). AceCS2 converts acetate to acetyl-CoA in the presence of ATP and its transcription is upregulated during ketogenic states such as starvation or diabetes. SIRT3 deacetylates and hence activates AceCS2 at lysine 642 providing a potential mechanism whereby SIRT3 could play a regulatory role in energy production and utilisation under certain conditions (Hallows *et al,* 2006, Schwer *et al,* 2006). Generation of a transgenic SIRT3 knockout mouse confirmed that a variety of mitochondrial proteins exhibit marked hyperacetylation. However, despite this profound alteration in enzymatic structure no demonstrable effect on physiological phenotype was noted under normal and short-term fasting conditions (Lombard *et al,* 2007). SIRT3 has also been implicated in the evolution of the ketogenic response to fasting by its ability to deacetylate mitochondrial 3-hydroxy 3-methylglutaryl CoA synthase 2 (HMGCS2) the rate limiting step in β-hydroxybutyrate synthesis (Shimazu *et al,* 2010). Hirschey *et al* (2010) report SIRT3 levels in liver and brown adipose tissue are increased in response to fasting. Furthermore, SIRT3 null mice exhibit hallmarks of fatty acid

oxidation disorders during fasting including reduced ATP levels and intolerance to cold exposure (Hirschey *et al,* 2010). It therefore, appears that SIRT3 may be involved in response to states of energy limitation when it can control the rate of ketosis and incorporate acetate into central metabolism where it can be used as an energy source.

1.4.5.2 Sirtuin 4

SIRT4 is found in mitochondria and unlike the sirtuin proteins already discussed it possesses ADP-ribosyltransferase activity, as demonstrated by the fact that mice lacking SIRT4 show no significant change in mitochondrial protein acetylation levels (Michishita *et al,* 2005, Lombard *et al,* 2007). SIRT4 has been implicated in amino acid-stimulated insulin secretion from pancreatic β-cells due to its ability to ADP-ribosylate and inhibit glutamate dehydrogenase (GDH). This enzyme controls the conversion of glutamate into α-ketoglutarate, a component of the citric acid cycle. By doing so it increases the ATP:ADP ratio a key stimulant of insulin secretion. Knockdown of SIRT4 results in upregulated amino-acid stimulated insulin secretion by increasing GDH activity (Haigis *et al,* 2006, Ahuja *et al,* 2007). SIRT4 may also play a role in the response to CR. Levels of SIRT4 are noted to decrease during CR corresponding with an increased GDH activity and hence insulin secretion from β-cells isolated from CR mice. Thus SIRT4 may play a role in changing the main stimulus to insulin secretion from carbohydrate to amino acids during CR (Haigis *et al,* 2006). A further recently identified function of SIRT4 is the capacity to oxidise fatty acids in liver and muscle cells. SIRT4 was knocked down both *in vivo* and *in vitro* resulting in an increase in hepatic mitochondrial and fatty acid oxidation gene expression (Nasrin *et al,* 2010). These findings again correlate with the potential that inhibition of SIRT4 during CR or periods of fasting controls entry into alternative means of energy production.

1.4.5.3 Sirtuin 5

Initial studies mapped the human SIRT5 gene to chromosome 6p.23 and found that it was particularly well expressed in cardiac myocytes and lymphoblasts, however further studies have demonstrated high expression levels in brain, liver, kidney, skeletal muscle and testis (Mahlknecht *et al,* 2006a). The first target of SIRT5 described was cytochrome C, a protein found in the mitochondrial inter-membrane space with a role in oxidative metabolism and apoptosis (Schlicker *et al,* 2008). The first *in vivo* target identified was recently uncovered as carbamoyl phosphate synthetase I (CPS I). This enzyme plays a key

role in the in the urea cycle which is responsible for the detoxification and excretion of the ammonia produced during the breakdown of amino acids. SIRT5 deacetylates and increases activity of CPS I, a response which is seen during conditions of fasting when the levels of NAD^+ increase in isolated mouse hepatocytes. Thus an adaptive mechanism by which nitrogenous waste, which is particularly abundant when amino acids are utilised as an energy source is induced. This was confirmed by the fact high levels of ammonia were recorded in fasted SIRT5 KO mice. These findings were also evident in mice chronically calorie restricted and fed high protein diets (Nakagawa *et al,* 2009, Nakagawa & Guarente 2009). It is therefore tempting to speculate that SIRT5 may contribute to the control of life span due to its ability to aid in the adaptation to alternative energy sources.

1.4.6 SIRTUIN 6

Intensive investigations into the function of SIRT6 have elucidated that its main function concerns DNA repair and maintenance of genomic integrity. It is located on chromosome 19 at position 13.3 and seems to possess both NAD^+ dependent deacetylase as well as ADP-ribosyl transferase activity. Expression levels are noted to be highest in thymus, skeletal muscle and brain (Liszt *et al,* 2005, Mahlknecht *et al,* 2006b, Pan *et al,* 2011). Initial clues regarding SIRT6 function came from knockout studies. $SIRT6^{-/-}$ mice were small, displayed profound abnormalities including lymphopenia, loss of subcutaneous fat, lordokyphosis, and severe metabolic defects including hypoglycaemia eventually dying at around four weeks (Mostoslavsky *et al,* 2006). These features are consistent with a premature ageing phenotype.

1.4.6.1 SIRT6 Maintains Genomic Integrity

SIRT6 plays an important role in the maintenance of genomic integrity and more specifically DNA damage repair. Analysis of MEFs isolated from $SIRT6^{-/-}$ mice revealed striking chromosomal aberrations and increased sensitivity to radiation damage. Furthermore they exhibited reduced capacity for BER when exposed to agents known to elicit DNA damage repaired by this pathway (Mostoslavsky *et al,* 2006). Recent evidence has also implicated SIRT6 in promoting DSB repair by promoting DNA-dependant protein kinase (DNA-PK) (McCord *et al,* 2009, Kaidi *et al,* 2010). Further work has also defined a role for SIRT6 in the maintenance of telomeric function. Cells deficient in SIRT6

displayed a senescence phenotype with increased markers of senescence and DNA damage response at telomeres. In addition there was evidence of increased chromosome end-to-end fusions indicating defective telomere function. The mechanism of telomere dysfunction is thought to be mediated through alteration in acetylation at H3K9 (Michishita *et al,* 2008b). It is, therefore clear from the results of these studies that SIRT6 is firmly established as a key mediator of genomic integrity with the ability to repair DNA damage of different types. As is evident from the original knockout mouse model this has fundamental implications for the ageing process.

1.4.6.2 SIRT6 & Metabolic Response

The potential role of SIRT6 in glucose homeostasis became apparent from examination of the SIRT6 knockout mice. These animals are hypoglycaemic from day 12 onwards to the extent that by day 24 glucose levels are undetectable with serum IGF-1 levels concomitantly reduced (Mostoslavsky *et al,* 2006). The mechanism behind this profound hypoglycaemia has recently been elucidated by Zhong and colleagues (2010). SIRT6 deficiency causes an upregulation of glucose uptake and switch in glucose metabolism to glycolysis from mitochondrial respiration, an effect caused by the SIRT6 mediated co-repression of HIF-1α, which is a key factor of the cellular adaptation to nutrient and hypoxic stress (Zhong *et al,* 2010, Zhong& Mostoslavsky 2010). This model predicts that in times of nutrient stress, for example CR, that SIRT6 would be inactivated triggering a HIF-1α dependant metabolic switch favouring glycolysis. This contradicts evidence produced by Kanfi *et al* (2008) suggesting that SIRT6 levels increase during CR (Kanfi *et al,* 2008b). This response is more in keeping with that expected for SIRT1 where it is seen to increase in response to nutrient deprivation (Cohen *et al,* 2004b). It may be that different sirtuins have evolved to function in contrasting scenarios.

1.4.6.3 SIRT6 & NF-κB

An intriguing discovery was that of Kawahara *et al* (2009), who show that SIRT6 functions at chromatin to attenuate NF-κB signalling. This is achieved by two main mechanisms firstly; SIRT6 interacts directly with the NF-κB RelA subunit and secondly by deacetylating H3K9 at NF-κB target gene promoters. Interaction between NF-κB and SIRT6 is implicated in normal and premature ageing as haploinsufficiency of the NF-κB subunit RelA rescues the premature ageing phenotype of SIRT6 deficient mice (Kawahara *et al,* 2009).

1.4.7 SIRTUIN 7

There is a relative paucity of information regarding the physiological function of SIRT7 in comparison with the other human SIRT proteins. The genomic locus has been shown to reside at 17q25.3. Expression levels of SIRT7 appear to be highest in blood and CD33+ bone marrow precursor cells as well as other rapidly dividing tissues such as liver, spleen and testis (Voelter-Mahlknecht *et al*, 2006).

Initial studies investigating the potential function of SIRT7 demonstrated that it co-localises to the nucleolus where it interacts with RNA polymerase Pol I, increasing its transcription and activity. Cells expressing SIRT7-shRNAs were significantly reduced in number, with further assays indicating cellular depletion of SIRT7 leads to apoptosis (Ford *et al*, 2006). Thus SIRT7 is implicated in the regulation of cell growth by driving ribosome biogenesis in response to fluctuating metabolic conditions. Grob *et al*, have also reported a role for SIRT7 in the resumption of rDNA transcription during telophase (Grob *et al*, 2009). Contrary to these findings studies using a SIRT7 knockout mouse model and harvested cells, have demonstrated anti-proliferative effects of SIRT7. MEFs derived from the SIRT7 knockout mouse model demonstrated increased viability under both normal and pro-oxidative conditions. (Vakhrusheva *et al*, 2008a). SIRT7 knockout mice display reduced mortality with evidence of myocardial hypertrophy and inflammatory cardiomyopathy. In addition, SIRT7 interacts with p53 resulting in its deacetylation corresponding to hyperacetylation of p53 and increased apoptosis in the myocardium of mutant mice (Vakhrusheva *et al*, 2008b).

1.4.8 Sirtuins in Mammalian Ageing, Calorie Restriction & the MTR

As outlined in the previous section there is an ever expanding body of evidence linking sirtuin expression to a complex array of biochemical and ultimately physiological processes, progressive dysfunction of which typify normal and abnormal organismal ageing.

1.4.8.1 Ageing & Lifespan Regulation

SIRT1 has been implicated in a number of different facets of the ageing process. For example, Chua *et al* (2005) reported that SIRT1 deficient MEFs have a dramatically increased resistance to replicative senescence, essentially becoming immortalised (Chua *et al,* 2005). Sasaki *et al* (2006) analysed SIRT1 levels from human lung fibroblasts and MEFs with normal, accelerated and delayed ageing. SIRT1 decreased with serial passage of cells and decreased rapidly in prematurely senescent cells. Furthermore at the animal level, SIRT1 decreased in the tissues in which mitotic activity decreases with age. This loss was accelerated in mice with an accelerated ageing phenotype (Sasaki *et al,* 2006).

When overall survival is taken into consideration results are variable. With both normal and prolonged lifespan reported in SIRT1 under-expressing transgenic mouse models under both normal and CR conditions (Boily *et al,* 2008, Li *et al,* 2008). The importance of SIRT1 in overall cellular function is underlined by the fact that mice in which the SIRT1 gene is completely knocked out rarely survive past the post-natal period, a response associated with p53 hyperacetylation (Cheng *et al,* 2003)

Evidence implicating sirtuins expression in human lifespan is sparse. However, it has been demonstrated that under-expression of human SIRT3 seems to be detrimental for longevity. Bellizi *et al* (2003) analysed the allele frequency of a VNTR polymorphism, which was shown to exhibit enhancer activity at the SIRT3 gene. The specific allele lacking enhancer activity was almost completely lacking in male study participants over the age of 90 (Rose *et al,* 2003, Bellizzi *et al,* 2005). Put together, the results of all these studies implicate the sirtuins in the control of longevity. This effect is likely to result from the ability of the sirtuins to maintain a fine balance between cellular resistance to stress, apoptosis and the promotion of efficient DNA repair.

1.4.8.2 Calorie Restriction

CR has been shown to extend lifespan in a number of different model systems (Imai & Guarente 2010). The adaptive responses induced by calorie restriction in mammalian models includes improved insulin sensitivity, reduced levels of glucose, cholesterol and free fatty acids along with reduced fat mass and increased levels of activity (Anderson & Weindruch 2010). A number of avenues of investigation have been followed leading to the

assumption that the responses induced by CR are mediated in part by the sirtuins and in particular SIRT1.

1. Expression of SIRT1 is induced in calorie restricted rats, an effect dependent on SIRT1 deacetylation of Ku70, inhibiting stress-induced apoptosis (Cohen *et al*, 2004b, Kanfi *et al*, 2008a).

2. Increased activity induced by CR is dependent on adequate SIRT1 expression (Chen *et al*, 2008).

3. Transgenic mice over-expressing SIRT1 display a number of phenotypic responses similar to those seen in CR mice (Bordone *et al*, 2007).

4. Treatment of regular chow fed mice with the SIRT1 activator resveratrol induces a transcription profiles similar to that of CR (Pearson *et al*, 2008).

5. Under-expression of SIRT1 specifically in the brain results in mutant mice who display defects in the endocrine and behavioural responses to CR (Cohen *et al*, 2009).

Clearly there exists a role for SIRT1 in the response to CR however both SIRT3 and SIRT6 have similarly been implicated. SIRT6 levels are increased in cultured cells, fasted mice and calorie restricted rats, an effect mediated by protein stabilisation as opposed to increased mRNA expression (Kanfi *et al*, 2008a). Two recent studies have implicated SIRT3 in the response to CR. Qui *et al* (2010) have demonstrated that SIRT3 is required for the protective effect of CR on levels of oxidative stress. Furthermore, reduction of cellular ROS levels is dependent on SIRT3 mediated deacetylation and hence augmentation of the activity of SOD2 (Qiu *et al*, 2010). Complementing these findings Someya and colleagues (2010) report that SIRT3 is required for the CR mediated prevention of age related hearing loss in transgenic mice. Again, this effect is mediated by SIRT3 dependent enhancement of the mitochondrial anti-oxidant defence system through regulation of isocitrate dehydrogenase 2 (Idh2) (Someya *et al*, 2010).

1.4.8.3 Sirtuins & the MTR

As mentioned previously the MTR trinity (Mitochondrion Telomere and Ribosome biogenesis) could provide a rationale paradigm whereby DNA damage is sensed and repaired. When the sub-cellular localisation and cellular function of the sirtuins are considered it is no surprise that they are considered the linchpin of the MTR hypothesis Each of the sirtuins could conceivably play a role in the MTR controlled stress response and it may be that there is interaction between sirtuins. As mentioned previously one of the defining features of sirtuin kinetics is the fact that NAD^+ is an essential cofactor for the activity of the majority of the sirtuins. This provides a plausible link between the metabolic state of the cell and the response to stress. SIRT1 is likely to be a key player in the overall response given its wide ranging function. Its ability to deacetylate p53 is likely to be crucial in the response mechanism. Via p53 SIRT1 has been shown to modulate cellular senescence and apoptosis in a number of models (Langley *et al,* 2002, Chua *et al,* 2005). SIRT1 has also recently been shown to attenuate ageing associated telomere attrition; hence SIRT1 could also play a role in determining the telomere associated DNA damage response (Palacios *et al,* 2010). Whilst SIRT6 plays a crucial role in the propagation of a specialised telomeric chromatin state essential for normal telomere function (Michishita *et al,* 2008b). The key roles of the mitochondrial sirtuins in metabolism and hence energy production are essential to fuel the damage response. However, this could be a double-edged sword as increase fuel utilisation with concomitantly increased generation of ROS could accelerate telomere attrition and initiate the DNA damage response. The interaction of the mitochondrial sirtuins, in particular SIRT3, with intermediaries of metabolism and anti-oxidant mechanisms is likely to be crucial in balancing the response (Schwer *et al,* 2002, Qiu *et al,* 2010). Ribosome biogenesis is one of the most energy consuming cellular processes, hence a balancing act where adaptation to cellular energy status is essential. SIRT1 has been shown to be essential for this response as part of the eNoSC (energy dependant nucleolar silencing complex) in combination with nucleomethylin and SUV39H1. This complex senses energy status, controls rRNA transcription and establishes silent chromatin at the rDNA locus (Murayama *et al,* 2008). Further control over ribosomal gene transcription is elicited by SIRT7,

Aberrancy of any of the components of the MTR could lead to dysregulation of any of the effector pathways resulting in a reduced capacity to sense and repair genotoxic damage,

with the ensuing potential of accelerated biological ageing and concomitantly associated condition such as cancer.

1.4.9 Sirtuins & Cancer

When the myriad of cellular processes in which the sirtuins have been implicated are considered it is no surprise that aberrancy of their expression is implicated in the process of cancer development and progression. This is particularly important in the case of SIRT1 which has an intimate relationship with p53, FOXO and many other factors not only fundamental in regulating the DNA damage response and the fate of a cell in stress, but ultimately protecting the cell from neoplastic transformation i.e. tumour suppressors. Rationalising the relationship between the sirtuins and tumourigenesis is complex. Currently the literature attempting to delineate the relationship between the sirtuins, and in particular SIRT1 and the cancer process is divergent with some studies claiming a tumour suppressor and some a tumour promoter role.

1.4.9.1 SIRT1 & Cancer

SIRT1 as a Tumour Promoter

It has been purported that SIRT1 could promote cancer formation and increase cancer risk by virtue of its ability to negatively regulate mediators known to act as tumour suppressors such as p53. However the relationship is not straightforward with complex interacting feedback loops regulating SIRT1, and its effect over a number of pro- and anti-oncogenic transcription factors and non-histone proteins. In order to be classed as a positive regulator of neoplastic transformation over-expression of SIRT1 in transgenic mice should increase the rate of detection of spontaneous cancers. However, although such mouse models show improvements in metabolic parameters consistent with the role of SIRT1 in metabolic pathways no increase in tumour development has been detected as yet (Banks *et al*, 2008, Pfluger *et al*, 2008).

In order to be recognised as a potential tumour promoter expression levels in isolated and cultured cells would have to be reflective of this hypothesis. Consistent with this SIRT1

has been shown to be upregulated in human cancer specimens and cells of different histological subtype (Table 1.6).

Table 1.6: Table summarising SIRT1 in cancer tissue expression studies.

Cancer Type	*Specimen*	*Method of Analysis*	*Comments*	*Reference*
Acute myeloid Leukaemia (AML)	Isolated and cultured primary AML blasts	RTq-PCR	Comparison with control cells ($CD34^+$ progenitors, peripheral blood mononuclear cells)	(Bradbury *et al,* 2005)
Human prostate	Tumour specimen	IHC	Comparison with uninvolved cells.	(Huffman *et al,* 2007)
Human prostate	Tumour specimen	IHC	Comparison with normal adjacent prostate tissue.	(Jung-Hynes *et al,* 2009)
Skin	Squamous cell, basal cell, Bowens disease, actinic keratosis biopsies	IHC	Comparison with 20 normal skin biopsies	(Hida *et al,* 2007)
Colorectal	Surgical specimens & CRC cell lines	IHC	Comparison with adjacent normal mucosa	(Stunkel *et al,* 2007)
Gastric	Surgical specimens post-gastrectomy	IHC	No direct comparison made but low expression in normal samples	(Cha *et al,* 2009)
Ovarian	Tumour samples	IHC	Increased expression in malignant compared with benign tumours.	(Jang *et al,* 2009)

SIRT1, p53 & Cancer

The ability of SIRT1 to deacetylate and therefore inhibit p53 activity lead investigators to assume SIRT1 expression would increase the risk of neoplastic transformation. Indeed overexpression of SIRT1 has been demonstrated to repress p53-dependant cell cycle arrest and apoptosis in response to DNA damage and oxidative stress, with the overall effect of promoting tumour growth (Luo *et al,* 2001, Vaziri *et al,* 2001, Cheng *et al,* 2003, Brunet *et*

al, 2004). Fine control of the p53-SIRT1 axis is essential for normal response to stress and the ageing process. A variety of control mechanisms have evolved to regulate the level of SIRT1 controlled acetylation level of p53 and hence its activity. One such strategy is the interaction between p53 and microRNA 34a (miR34a). The miR34a family regulates cell cycle progression, senescence and apoptosis and, importantly its transcription can be activated by p53. Moreover, it is suggested it functions as tumour suppressor *in vivo* (Yamakuchi & Lowenstein 2009). miR34a can also bind to the 3'-UTR of SIRT1 mRNA to repress its translation. Consistent with this finding over-expression of miR34a reduced SIRT1 activity in human colon cancer cells and enhanced p53-dependent apoptosis, an effect not observed in cells lacking p53. Importantly, over-expression of SIRT1 rescued colon cancer cells from miR34a induced apoptosis (Yamakuchi *et al,* 2008). Thus in a cellular environment where SIRT1 is over-expressed the tumour suppressor effect of p53 and miR34a is abrogated by disruption of the SIRT1-p53-miR34a axis.

SIRT1, HIC1 & p53

Hypermethylated in cancer-1 (HIC1) is a commonly mutated tumour suppressor gene. This is particularly important when the interaction between HIC1, SIRT1 and p53 is considered. HIC1 and SIRT1 form a transcriptional repression complex which binds directly to the SIRT1 promoter, repressing its transcription. In cancer cells mutated and hence inactivated HIC1 results in up-regulated SIRT1 expression, leading to augmented deacetylation and inactivation of p53, thus allowing cells to bypass apoptosis and survive DNA damage (Chen *et al,* 2005a). The authors propose a HIC1-SIRT1-p53 regulatory loop that in normal circumstances controls growth arrest and apoptosis in response to DNA damage. This regulatory loop gains particular importance during organismal aging when the HIC1 promoter becomes progressively hypermethylated, promoting survival of aging cells but increasing cancer risk (Chen *et al,* 2005a).

SIRT1, DBC1 & p53

Deleted in Breast Cancer-1 (DBC1) is a protein with purported tumour suppressor function resulting from its effect on cell proliferation, apoptosis and histone modification (Kim *et al,* 2009). DBC1 is a negative regulator of SIRT1 which forms a stable complex with SIRT1 inhibiting its activity, as expected this leads to increased p53 acetylation and up-regulation of p53 function. Knockdown of DBC1 by RNAi promoted SIRT1 mediated deacetylation of p53 and inhibited apoptosis induced by genotoxic stress (Kim *et al,* 2008,

Zhao *et al,* 2008). These data indicate that increased SIRT1 expression or reduced DBC1 activity increase cancer risk by inhibiting p53.

SIRT1, FOXO & Cancer

The ability of the FOXO factors to induce cell cycle arrest, DNA repair and apoptosis makes them attractive candidates as tumour suppressors. Inactivation of FOXO could lead to dysfunctional cell cycle checkpoints leading to neoplastic transformation. Consistent with this over-expression of FOXO reduces tumourigenicity in a number of experimental models (Greer & Brunet 2005, Greer & Brunet 2008). Ford *et al* (2005) used RNAi to determine if either FOXO3 or FOXO4 can influence the apoptotic consequences of SIRT1 inhibition in human colorectal cancer cells. Co-silencing experiments revealed that FOXO4 but not FOXO3 rescues SIRT1 siRNA-treated cells from apoptosis, inferring that FOXO4 is essential for apoptosis in these cancer cells (Ford *et al,* 2005). Thus the overall effect of the interaction between the FOXO transcription factors and SIRT1 may be to promote tumourigenesis by a shift from pro- to anti-apoptotic pathways with promotion of cell cycle progression.

SIRT1 as a Tumour Suppressor

For a gene product to be considered a tumour suppressor a number of key criteria have to be met. Firstly, over-expression in cell culture should induce cell growth arrest. Secondly, there should be evidence of deleterious point mutations or gene deletions in human cancer subtypes and finally there should be evidence of under-expression or hypo-activity in isolated cancer cells (Brooks & Gu 2009). There is accumulating evidence from expression studies and *in vitro* work in specific cancer types that SIRT1 can act in a tumour suppressive fashion.

Wang *et al* (2008) have extensively investigated the expression of SIRT1 in a number of tumour types. They report that levels of SIRT1 were lower than normal control tissue in glioblastoma, bladder, prostate and ovarian carcinoma. These results were validated using western blot analysis, where there was reduced expression in breast and hepatocellular carcinoma with equivalent expression levels between lung, colon, stomach, bladder and skin cancer specimens, and their respective control tissue samples (Wang *et al,* 2008a).

Stimulation of SIRT1 deficient MEFs by chronic oxidative stress resulted in diminished upregulation of the senescence mediator $p19^{ARF}$ and its downstream target p53, with a concomitant extension of replicative lifespan. These results raise the possibility that SIRT1 may exert a negative regulatory role on oncogenesis by inducing the expression of tumour suppressor genes and senescence. However, the same study failed to show any change in $p19^{ARF}$ expression or progress through the cell cycle in SIRT1 deficient MEFs subjected to viral oncogene expression (Chua *et al,* 2005).

Two main studies have utilised transgenic models to provide evidence that it is the ability of SIRT1 to promote genomic integrity that may explain in part its tumour suppressive effect. Wang *et al* (2008) developed double heterozygotic $SIRT1^{+/-}$; $p53^{+/-}$ mice, which developed a high incidence of tumours with evidence of aneuploidy and chromosomal aberrations consistent with severe genetic instability. (Wang *et al,* 2008a). Oberdoerffer *et al* (2008) developed a transgenic murine $p53^{+/-}$ model in which SIRT1 was over-expressed. This combination resulted in decreased incidence of thymic lymphoma and increased survival following exposure to γ-irradiation. Further investigation using isolated MEFs showed that SIRT1 dissociates from its original loci to DSBs. Again this was shown to be dependent on H2γX, but also on intact ATM signalling pathways (Oberdoerffer *et al,* 2008). These studies provide further evidence of a tumour suppressive function of SIRT1 via its role in the maintenance of genomic integrity and prevention of potentially oncogenic chromosomal aberrations.

SIRT1 as a Pro-apoptotic Tumour Suppressor

An interaction exists between SIRT1 and survivin in BRCA1 association breast cancers whereby, SIRT1 suppresses survivin. Survivin is an inhibitor of apoptosis and is over-expressed in many tumour types, making it an attractive chemotherapeutic target. BRCA1 associated cancers have low SIRT1 expression levels with high levels of survivin. BRCA1 is found at the SIRT1 promoter in cells expressing wild type BRCA1 and increases SIRT1 expression which in turn inhibits survivin by deacetylation. Furthermore, treatment with resveratrol inhibits the growth of BRCA1 deficient tumours both *in vitro* and *in vivo* (Wang *et al,* 2008b).

As mentioned previously NF-κB is a ubiquitous mediator of the cell, cycle, growth, angiogenesis and apoptosis. Yeung *et al* (2004) demonstrated that SIRT1 over-expression or treatment with resveratrol sensitised non-small cell lung cancer (NSCLC) cells to apoptosis when stimulated by TNF-α, by deacetylating the RelA/p65 subunit of NF-κB (Yeung *et al*, 2004).

SIRT1 Activation by Resveratrol

Resveratrol (3,5,40-trihydroxystilbene) is a naturally occurring phytoalexin found in grapes, peanuts and various berries. It has gained popular attention due to its purported health benefits stemming from its anti-oxidative capacity. It is thought to some degree to explain the French paradox whereby rates of coronary artery disease are lower in France compared many other western countries despite high levels of saturated fat intake. This effect is thought in part to be mitigated by higher levels of red wine and hence resveratrol consumption (Lippi *et al,* 2010). However, a number of other potential health benefits have been attributed to resveratrol, particularly pertinent is its beneficial effect in cancer. The mechanism by which resveratrol imparts its beneficial effects is matter of great debate, however there are well defined effects on sirtuin expression, particularly an up-regulation of SIRT1 levels (Howitz *et al*, 2003).

The anti-proliferative effect of SIRT1 activation by resveratrol has been investigated in a number of experimental contexts. It was first demonstrated to possess anti-carcinogenic effects in 1997, on a murine model of skin carcinogenesis (Jang *et al,* 1997). However, it also has beneficial effects on tumour growth in a mouse model of colorectal carcinogenesis (Firestein *et al*, 2008), in BRCA1 mutated breast cancer models (Wang *et al*, 2008b) and is pro-apoptotic in non-small cell lung cancer cells (Yeung *et al,* 2004). The beneficial effect of resveratrol on a murine model of skin cancer is partly SIRT1 dependent (Boily *et al*, 2009).

1.4.9.2 SIRT2 & Cancer

SIRT2 is a tubulin deacetylase required for normal mitotic progression and control of mitotic checkpoint function to ensure chromosomal fidelity (North *et al*, 2003, Inoue *et al*, 2007, Inoue *et al,* 2009). Hence is reported to be a negative regulator of cellular proliferation. Consistent with this SIRT2 expression is found to be reduced in human

glioma tissue samples and cell lines, indicating a potential tumour suppressor effect (Hiratsuka *et al*, 2003).

1.4.9.3 SIRT3, SIRT4, SIRT5 & Cancer

It is becoming more apparent that the 'mitochondrial' sirtuins have complex cellular functions not just limited to their original description as regulators of metabolism. The first supportive evidence demonstrated a link between SIRT3 expression and node positive breast cancer (Ashraf *et al*, 2006). More recently Kim *et al* (2010) provided evidence supporting a tumour suppressor role for SIRT3. $SIRT3^{-/-}$ mice develop ER/PR-positive mammary tumours, whilst *in vitro* transformation of $SIRT3^{-/-}$ MEFs by oncogene expression is reversed by superoxide dismutase (Kim *et al*, 2010).

There are as yet no studies directly linking either SIRT4 or SIRT5 to cancer initiation, progression or metastasis. This situation will almost certainly change in the future as the knowledge base surrounding these sirtuins expands.

1.4.9.4 SIRT6 & Cancer

SIRT6 is responsible for maintenance of genomic integrity, an effect mediated by its ability to control BER via its ability to complex with DNA-PK and modulate telomeric chromatin (Mostoslavsky *et al*, 2006, McCord *et al*, 2009, Michishita *et al*, 2009). Clearly aberration of SIRT6 expression could therefore lead to genomic instability, a hallmark of a number of cancer subtypes. In contrast, lack of SIRT6 might provide a growth advantage for tumour cells as SIRT6 deficient cells are more resistant to apoptosis when exposed to hypoxia, an effect mediated by HIF-1α, which is up-regulated in many primary cancers (Zhong *et al*, 2010).

1.4.9.5 SIRT7 & Cancer

Potential for the role of SIRT7 in cancer comes from different lines of research. The region of the genome in which SIRT7 resides is frequently implicated in acute leukaemia and lymphoma. (Voelter-Mahlknecht *et al*, 2006). Meanwhile SIRT7 expression is reported to be increased in human papillary thyroid cancer, and also in breast cancer tissue when compared with control and non-malignant tissue (Ashraf *et al*, 2006, de Nigris *et al*, 2002, Frye 2002).

1.5 Statement of Research Aims

With the advancing age of the general population and the strong risk elicited by chronological age on cancer risk, the incidence of cancer is only likely to increase in the next few decades. It is therefore intuitive that delineating the molecular mechanisms which influence each of the processes could lead to the development of novel targets for intervention. As presented above accelerated telomere attrition and aberrant sirtuin expression have shown a clear and consistent link with the cancer process in published studies. However, some controversies and dichotomies exist. Some studies have presented counter-intuitive evidence of long telomeres associated with cancer risk. Whilst, studies have still have not reconciled the role of the sirtuins as either tumour promoters or suppressors. In addition studies have mainly focused on the molecular biology of the sirtuins in the neoplastic as opposed to the clinical significance of their expression. The overall aim of this study, therefore, is to determine the role and significance of telomere attrition and sirtuin relative expression in colorectal cancer. This will be achieved through a number of research questions:

1. Do patients with colorectal cancer display evidence of accelerated biological ageing in the form of telomere attrition?

2. Are peripheral blood leucocyte (PBL) telomere lengths reflected in associations with, pro-inflammatory cytokines (IL-6/IL-10), CRP and levels of circulating factors involved in redox control (fetuin-A, antioxidant vitamins, trace elements) inthe CRC patient cohort?

3. Does PBL telomere length predict adverse pathological or prognostic factors?

4. Is there a difference in telomere length between colorectal tumour and normal adjacent tissue, does tissue telomere length correlate with pathological features?

5. Is aberrancy of sirtuin expression evident in colorectal cancer, as indicated by differential relative expression between tumour and adjacent normal tissue?

6. Do sirtuin expression levels predict adverse pathological or prognostic tumour features?

7. Is there any relationship between sirtuin expression and cellular ageing indicated by telomere length at the tumour level?

8. Finally, does sirtuin expression correlate with a variety of commonly recorded biochemical and haematological variables, particularly those involved in systemic inflammation?

The work carried out in this thesis therefore aims to place the biology of ageing and CRC in a clinical context, and by doing so provide robust information that may prove useful in the hunt for genuine new targets for anti-mitotic agents or markers of disease severity.

CHAPTER 2: MATERIALS & METHODS

As outlined in the aims section of this thesis there are two main components to this study: determination of differences in the biological age of colorectal cancer patients compared with controls using PBL TL and comparison of the relative transcriptional expression levels of SIRT1-7 between colorectal tumour and normal tissue. This therefore required two sources of study material from colorectal cancer patients, blood samples to harvest PBLs and extract DNA and tumour tissue to extract RNA.

2.1 Patient Recruitment & Sample Collection

2.1.1 PBL Group

This group was comprised of patients recruited prospectively from the Department of Colorectal Surgery, Glasgow Royal Infirmary. All patients were admitted for assessment and management of histologically proven colorectal adenocarcinoma. Blood samples (n=64) were collected for analysis during diagnostic workup. 10mls of whole blood was collected in EDTA tubes and immediately centrifuged. The resulting cellular component and buffy coat was stored at 4^{o}C until use in the telomere assay. The plasma was stored at -80^{o}C and used in the experiments to determine potential correlates of TL including redox control factors, markers of systemic inflammation and fetuin-A. Table 2.1 presents details of the patient clinical and pathological variables for this group (this group will be referred to as the CRC PBL group). Full ethical approval from the local NHS ethics committee was gained prior to the commencement of any sample collection (COREC 08/S0704/22).

Table 2.1: Table displaying baseline demographic and clinico-pathological variables for the CRC PBL telomere group.

		CRC PBL Group	
Characteristic		**Number/Total**	**%**
Sex	Male	33/64	51.56
	Female	31/64	48.44
Age	45-55	9/64	14.06
	56-65	17/64	26.66
	66-75	24/64	37.5
	76-85	14/64	21.88
Tumour Site	Colon	42/64	65.63
	Rectum	22/64	34.37
Duke's Stage	A	6/64	9.38
	B	23/64	35.94
	C	16/64	25.00
	D	18/64†	28.13
	Unknown	1/64	1.67
Tumour	T1	2/52	3.85
	T2	8/52	15.38
	T3	30/52	57.69
	T4	12/52	23.08
Nodes	N0	30/52	57.69
	N1	15/52	28.85
	N2	7/52	13.46
Metastasis	M0	47/52	90.38
	M1	5/52¶	9.62
‡Peterson Index	High Risk	13/48	27.18
	Low Risk	35/48	72.92
mGPS	0	42/64	65.63
	1	13/64	20.31
	2	9/64	14.06
	Unknown		
Smoker	Never	23/64	43.40
	Ever	20/64	37.74§
	Unknown	11/64	18.86
Carstairs Index	2	2/64	3.13
	3	8/64	12.5
	4	11/64	17.2
	5	7/64	11.0
	6	5/64	7.81
	7	28/64	43.75
	Unknown	3/64	

† Includes patients with in-operable disease and patients who underwent synchronous resection of their primary cancer and liver metastasis.
¶ Synchronous resection of liver metastasis.
‡ Pathological scoring system where score of 2 or more indicates high risk pathological features. Points awarded for vascular invasion (1), margin positivity (1), peritoneal breach (1) & tumour perforation (2)
§This compares with 58% of the control population who had 'ever' smoked.

2.1.2 Tissue Group

The second group utilised colorectal cancer tissue (tumour and normal) collected under the auspices of the local Biobank (NHS Greater Glasgow & Clyde), this group was used to determine relative transcriptional expression levels of SIRT1-7 and in addition, cancer tissue TL (Table 2.2). All measurements were performed in paired samples (tumour and adjacent normal tissue) to allow direct comparison. Colorectal cancer patients who were admitted for potentially curative surgery were approached pre-operatively by Biobank staff and full informed consent obtained for the collection and use of excess tissue for research purposes. A total of fifty five paired samples were available for RNA extraction. All samples were snap frozen in liquid nitrogen and stored at -80^{o}C until use. All tissue samples were validated by a consultant pathologist and deemed representative of the pathological specimen. Of the 55 samples, 32 paired tissue samples were available for additional DNA extraction and subsequent TL analysis (this group will be referred to as the CRC tissue group). Full ethical approval was gained for use of surplus Biobank tissue for use in research (COREC 08/S0704/42)

Table 2.2: Table displaying the patient characteristics of the full group of patients from tissue samples were collected and RNA extracted for use in the RT-PCR assay to determine SIRT1-7 relative expression and the 32 samples from which DNA was extracted to determine tissue telomere length.

		CRC Tissue Group			
		Sirtuin Experiment		Telomere Experiment	
Characteristic		**Number/Total**	**%**	**Number/Total**	**%**
Sex	Male	27/55	49.1	15/32	46.88
	Female	28/55	50.9	17/32	53.13
Age	<65	9/55	16.4	4/32	12.5
	65-75	24/55	43.6	15/32	46.9
	>75	22/55	40	13/32	40.6
Tumour Site	Colon	39/55	70.9	26/32	81.25
	Rectum	16/55	29.1	6/32	18.75
Duke's Stage	A	10/55	18.2	4/32	12.5
	B	27/55	49.1	16/32	50.0
	C	12/55	21.8	8/32	25.0
	D	6/55†	10.9	4/32†	12.5
Tumour	T1	0/55		0/32	
	T2	10/55	18.2	4/32	12.5
	T3	28/55	50.9	19/32	59.38
	T4	17/55	30.9	9/32	28.13
Nodes	N0	39/55	70.9	21/32	65.63
	N1	13/55	23.6	9/32	28.13
	N2	3/55	5.5	2/32	6.25
Metastasis	M0	49/55	89.1	28/32	87.5
	M1	6/55†	10.9	4/32†	12.5
Peterson Index‡	Low Risk	40/55	72.7	26/32	81.25
	High Risk	15/55	27.3	6/32	18.75
mGPS	0	31/55	56.4	18/32	56.25
	1	10/55	18.2	3/32	9.38
	2	6/55	14.5	6/32	18.75
	Unknown	6			
Smoker	No	28/55	50.9	5/32	15.63
	Yes	23/55	41.8	18/32	56.25
	Unknown	4		11/32	34.38

† synchronous resection of their primary cancer and liver metastasis.
‡ Pathological scoring system where score of 2 or more indicates high risk pathological features. Points awarded for vascular invasion (1), margin positivity (1), peritoneal breach (1) & tumour perforation (2)

2.1.3 Correlation with Patient Clinico-Pathological Factors

An attempt was made to correlate both PBL TL and sirtuin expression data with routinely available clinico-pathological parameters. Where possible biochemical, haematological and pathological data were extracted from a prospective database maintained by the Department of Surgery (details in individual results chapters). The Peterson Index (PI) was used as an additional measure to identify patients with pathologically more aggressive

disease and hence poorer outcome. Pathologically determined vascular invasion, margin involvement or serosal breach was allocated a score of 1, with tumour perforation scoring 2. A cumulative score of 0-1 indicates a low risk and 2-5 a high risk PI. A high risk PI suggests aggressive disease and has been shown to correlate with a poorer outcome from CRC in lymph node negative patients (Petersen *et al*, 2002).

2.1.4 Control Group

The control population used in the comparative study with CRC patients was comprised of subjects from the MRC West of Scotland Twenty-07 Cohort. Subjects used to form this cohort comprised of 1348 individuals aged either 57 (n=847) or 76 (n=501). This is a community-based cohort study designed to longitudinally investigate the social processes that produce or maintain inequalities in health and has been described in detail previously (Benzeval *et al*, 2009). The two age groups were specifically chosen to demonstrate that both the chronologically older group and an age adjusted combined group had longer telomeres than CRC patients. In addition to age, sex and smoking status was available for this cohort. None of the subjects in this group had been diagnosed with cancer at any stage.

2.2 Quantitative Real-Time PCR

Quantitative real-time PCR was utilised as the assay of choice in the analysis of PBL TL and also SIRT1-7 relative expression. Although the same basic technique was used in each of the assays different methods of quantification were used to give an estimation of TL and relative quantification of sirtuin gene transcription. These modifications along with the specifics of the reaction will be outlined in the relevant methods section. The essential steps of the reaction are however are now discussed.

Real-time PCR (RT-PCR) has a number of advantages over more rudimentary PCR techniques. The main advantage is the ability to detect PCR amplification during the early phases of the reaction. Basic end-point PCR techniques rely on agarose gels to detect the amplification product towards the end of the reaction leading to results which are imprecise, of low sensitivity and resolution. Accuracy is greatly increased by quantification at the earlier exponential phase of the PCR reaction.

The RT-PCR reaction assumes that there is a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. The reaction relies on a DNA polymerase with 5’ exo-nuclease activity of which there are several commercially available systems such as AmpliTaq Gold® TaqMan® system. The other key component to this reaction is a specific oligonucleotide probe which is specific for a DNA sequence between the forward and reverse primers. These probes are designed with a high energy dye at the 5’ end termed a reporter and lower energy dye at the 3’ end termed a quencher. When the probe is intact the reporter dye emission is suppressed by the quencher. However, when the probe is cleaved by the 5’ exo-nuclease the energy from the reporter molecule is released and sensed by a fluorescence sequence detection system (again a number of these are commercially available such as the ABI Prism Detection System). Thus with each cycle of the PCR reaction there is an increase in the fluorescence emission detected by the reporter dye, furthermore the reaction is specific at three levels, the complementary probe cleaved during the amplification reaction along with the forward and reverse primers.

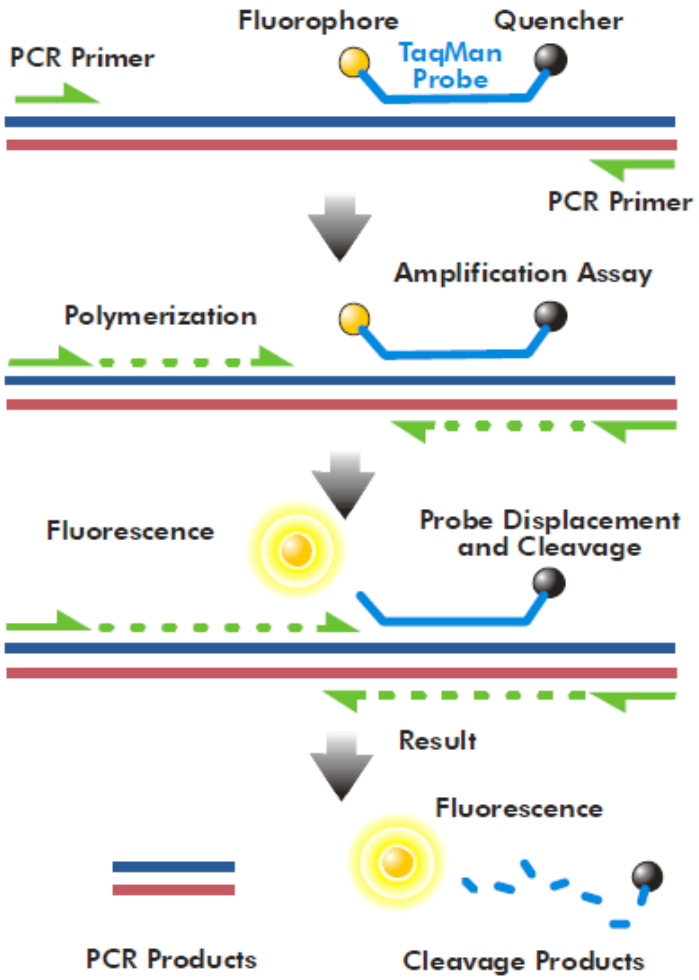

Figure 2.1: Schematic representation of the RT-PCR reaction involving the specific Taqman® probe and primer set with attached quencher and reporter (fluorophore) dyes. Taken from (Stratagene 2004)

Quantification of gene expression using RT-PCR requires normalisation of the reporter dye emission signal using a passive reference dye. This is incorporated into the PCR mastermix and is required to correct for fluctuations in fluorescence as a result of alterations in concentration or volume. The Rn (normalised reporter) value is the emission of the reporter dye divided by the emission of the normalised reporter. The ΔRn is the change in Rn between an untreated or early cycle sample (Rn-, no template control) and a sample containing a full complement of reaction components including the target (Rn+). The ΔRn reflects the initial level of target DNA, as the signal increases this indicates the amount of hybridised probe that has been degraded by the exo-nuclease. The Rn is used by the software of the detection system to define a baseline of fluorescence (usually determined from the initial cycles of the reaction) and a threshold which is the average standard deviation of Rn for the early PCR cycles and is set in the exponential phase of the reaction

when it is at its most efficient. Finally, the threshold cycle (Ct) is the cycle number at which fluorescence passes the fixed threshold. The Ct value is used in the final calculation of gene expression. Different methods of relative quantification were used in this study to determine PBL TL and sirtuin gene relative expression which will be detailed in due course.

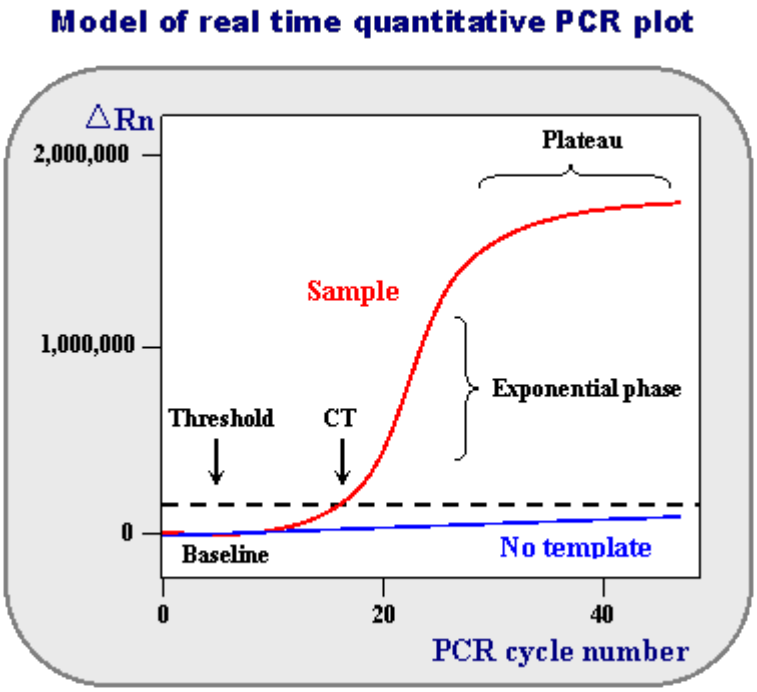

Figure 2.2: Schematic representation of the ideal RT-PCR reaction.

2.3 PBL and Tissue Telomere Length Determination and Correlation in Colorectal Cancer

2.3.1 Tissue Processing & DNA extraction

DNA was extracted from both blood and tissue using the Maxwell® automated purification system according to the manufacturer's instructions (Promega, WI, USA). Briefly, whole blood samples were spun down into cellular and plasma components. The erythrocytic component and buffy coat were thoroughly mixed and 300µl aliquoted into

the predispensed reagent cartridges. Cancer and normal tissue samples were thawed and 50mg added to the reagent cartridge. The DNA concentration and purity were quantified using the Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, USA). All DNA samples were validated on 0.5% agarose gel (Figure 2.3).

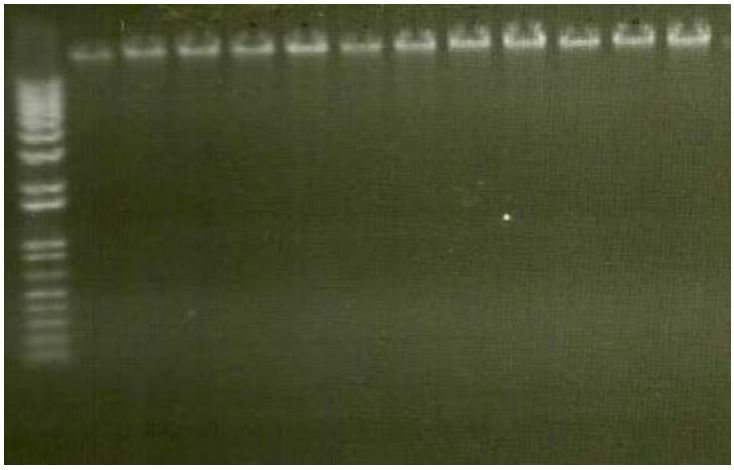

Figure 2.3: 0.5% TBE agarose gel demonstrating validation of the extracted DNA for use in the telomere length determination assay (300ng DNA used).

2.3.2 Telomere length Determination Using Real-Time PCR

Telomere lengths were determined from DNA extracted as outlined above from the PBLs of both the control and CRC group and from the CRC tissue (tumour and normal). This was achieved by Q-PCR using the method first described by Cawthon (Cawthon 2002). This method aims to determine sample DNA relative TL (relative T/S) by determining the ratio of telomere repeat copy number to single gene copy number and comparing this with a reference DNA sample of known relative TL. The telomere repeat to single copy gene ratio (T/S ratio) is proportional to the average TL. The quantity of telomere and single copy repeats are determined using the standard curve method where successive dilutions of the reference DNA are used to construct a standard curve which is then used to determine copy number. The gene used to determine single copy number was the ribosomal protein 36B4 gene.

2.3.2.1 Plate Construction

All samples were assayed in triplicate on 96-well plates. In order to determine the telomere copy number to single gene copy number pairs of plates were used to run each sample

using the telomere primer mix and the single gene primer mix, in this case the acidic ribosomal phosphate 36B4 was utilised. Telomere and 36B4 plates were constructed identically to include; a five point standard curve created from the stock solution of reference DNA (standards range from 100ng-3.13ng), reference DNA sample (positive control/calibrator 10ng/µl), no template negative control and sample DNA (7ng/µl) (Table 2.3 & Figure 2.4).

	1	2	3	4	5	6	7	8	9	10	11	12
A	SD1	SD1	SD1	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	SD2	SD2	SD2	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	SD3	SD3	SD3	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	SD4	SD4	SD4	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	SD5	SD5	SD5	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	SD6	SD6	SD6	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	SC	SC	SC	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample

Figure 2.4: 96 well plate layout for both telomere and 36B4 plates. SD1-6 = serial dilutions for standard curve construction. SC = control reference sample.

Table 2.3: Table displaying the reaction constituents for the telomere and 36B4 plates. Sample DNA was used at a concentration of 7ng/µl. (Primer sequences displayed in Figure 2.5)

Telomere Master Mix	Volume (µl)	36B4 Master Mix	Volume (µl)
SYBER green 2X master mix (Roche, Switzerland)	10	SYBER green 2X master mix (Roche, Switzerland)	10
Telo 1 primer	0.75	36B4 Fwd primer	0.4
Telo 2 primer	0.75	36B4 Rev primer	0.4
PCR grade H_20	3.5	PCR grade H_20	4.2
Sample DNA	5	Sample DNA	5
Total	20	Total	20

Analysis was performed on the Roche Light Cycler LC480 (Roche, Switzerland). The telomere-specific amplicon primer set was previously validated and the optimum concentration determined (Koppelstaetter *et al*, 2005) (Figure 2.5).

Tel 1, CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
Tel 2, GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

36B4 for, CAGCAAGTGGGAAGGTGTAATCC
36B4 rev, CCCATTCTATCATCAACGGGTACAA

Figure 2.5: Primer sequences for telomere and 36B4 genes.

All samples were heated to 95^{o}C for 5 minutes prior to plate construction and then subsequently run under the following conditions:

Telomere plates: 95^{o}C for 10 minutes (polymerase activation)

30 cycles of 95^{o}C for 5 seconds
59^{o}C for 10 seconds
72^{o}C for 2 minutes

Melt Sequence (1 cycle) 95^{o}C for 30 seconds
65^{o}C for 30 seconds
95^{o}C for 2 minutes

Cool Sequence (1 cycle) 40^{o}C for 10 minutes

36B4 plates: 95°C for 10 minutes (polymerase activation)

35 cycles of 95°C for 5 seconds
59°C for 15 seconds
72°C for 15 seconds

Melt Sequence (1 cycle) 95°C for 30 seconds
65°C for 30 seconds
95°C for 2 minutes

Cool Sequence (1 cycle) 40°C for 10 minutes

2.3.2.2 Data Analysis

The LightCycler LC480 software version 1.5 was used to generate standard curves and for subsequent quantification of the telomere and single gene copy number. This relative T/S value is the estimate of the relative TL of the sample in question. In order to make meaningful comparisons between the two plates (telomere and single copy) the efficiency of the PCR reaction of the two plates should be as similar as possible. This is determined by the slope of the standard curve, which was deemed acceptable if the slope was between -3.1 and -3.8 with a perfect slope = -3.2. An example of a typical generated standard curve is shown in Figure 2.6. The computer software then compares the sample quantities on the telomere plate with their counterparts on the 36B4 plate to give a T/S ratio, which is then normalised to the T/S of the reference DNA sample to give a relative T/S or telomere length for each DNA sample under investigation.

The intra-assay co-efficient of variation (CV) for the telomere plates was 0.43% and 0.15% for the 36B4 plates. The intra-assay CV was 17%.

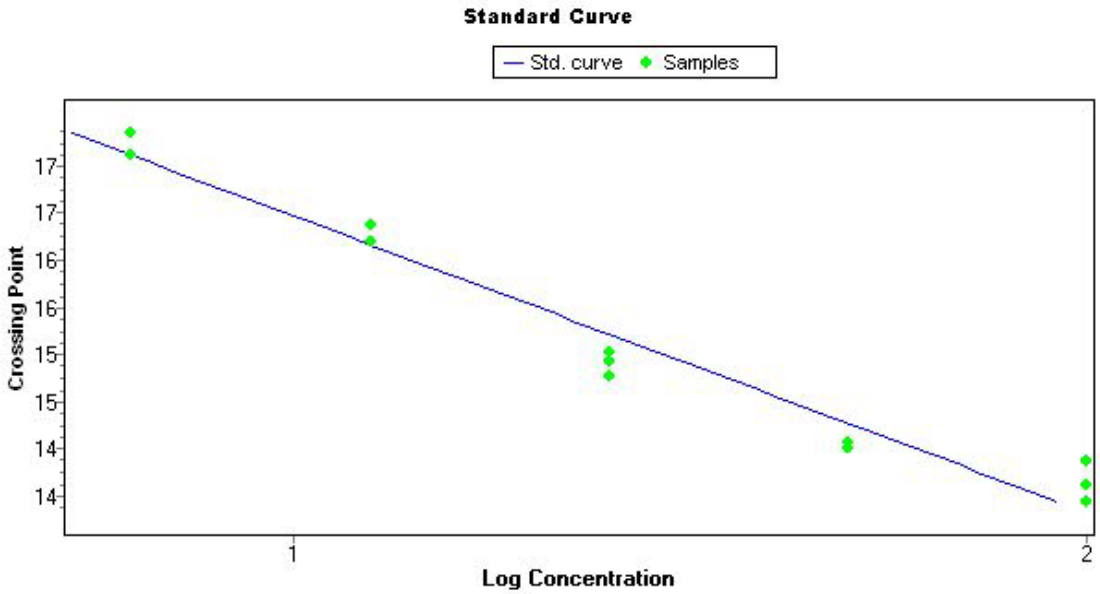

Figure 2.6: Shows the software generated standard curve. x-axis displays the logarithmic transformation of the individual standard concentrations. y-axis displays the crossing point which is analogous to the Ct and is the threshold cycle at which amplification products are first detected. The slope for this curve is -3.147.

2.3.3 Correlates of PBL Telomere Length

2.3.3.1 Measurement of Plasma Fetuin-A

Fetuin-A concentrations were measured from the plasma of blood samples of CRC PBL patients using a commercial Human Fetuin-A ELISA Kit (BioVendor R&D, Czech Republic). Fetuin-A was not measured in either the CRC tissue group or the control population. Samples were measured in triplicate. Absorbance of each sample was read by a microplate reader at dual wavelengths 405nm and 650nm, and sample concentrations were then calculated using the standard curve. All methodologies were carried out according to the manufacturer's instructions. The minimum detectable concentration was 0.35ng/ml.

2.3.3.2 Measurement of Markers of Systemic Inflammation

Systemic inflammation is known to predict poor outcome from colorectal cancer and is also intricately linked with aspects of biological ageing, in particular telomere attrition. For the CRC PBL and tumour patients, routinely available indices of inflammation (CRP, albumin, white cell, neutrophil and lymphocyte count) were measured using routine methods in the Departments of Haematology and Biochemistry, Glasgow Royal Infirmary. For the CRC PBL patients only plasma concentrations of interleukin (IL)-6, IL-10 and

vascular endothelial growth factor (VEGF) were measured using enzyme linked immunosorbent assay (ELISA) kits (Quantikine ELISA, R&D Systems, Huntingdon, UK). The minimum detectable concentrations were 2 pg/ml for IL-6, 4pg/ml for IL-10 and 5pg/ml for VEGF (these analyses were performed by Fiona Breckenridge, Dept of Biochemistry, Glasgow Royal Infirmary). The neutrophil to lymphocyte ratio (NLR) and modified Glasgow Prognostic Score (mGPS) was calculated for each patient thereby giving an estimation of systemic inflammation. mGPS is calculated by awarding a point for plasma albumin level under 35 mg/dl and C- Reactive Protein (CRP) under 10 mg/l thereby giving a score of 0,1 or 2. A score of 1 or 2 indicates higher levels of systemic inflammation and has been shown in numerous cancer types to correlate with poor cancer specific survival (Roxburgh& McMillan 2010).

2.3.3.3 Measurement of Redox Control Factors

The separated plasma from each blood sample was used to create a redox profile for each CRC PBL patient. Concentrations of antioxidant vitamins A (retinol) and E (α-tocopherol) and the carotenoids (lutein, lycopene, α- and β-carotene) were determined using a high performance liquid chromatography (HPLC) based assay as previously described (Talwar *et al*, 1998). Plasma was de-proteinised with alcohol containing internal standards and extraction of the analytes was performed using hexane. Analysis was carried out using reversed phase–HPLC (5 μm microbore, Phenomenex, Macclesfield, UK) and dual wavelength monitoring (Waters, MA). The 95% normal reference intervals for the above assays as established in our laboratory were as follows: retinol (1.0-2.8 μmol/l), α-tocopherol (14-39 μmol/l), lutein (82-202 μg/l), lycopene (100-300 μg/l), α-carotene (14-60 μg/l) and β-carotene (92-312 μg/l). These analyses were performed in the laboratory of Dr Dhinesh Talwar, Department of Biochemistry, Glasgow Royal Infirmary.

2.3.3.4 Measurement of Vitamin D

Plasma Vitamin D (25-OHD) concentration was measured from collected and separated samples using an automated solid phase extraction procedure with subsequent HPLC- mass spectrometry technique. The lower limit of sensitivity was 4 nmol/L (assay performed in the laboratory of Dr Dhinesh Talwar, Department of Biochemistry, Glasgow Royal Infirmary.

2.3.4 Statistical Analysis

All clinical, pathological and biochemical data were displayed either categorically or as median with inter-quartile range (IQR). TL was analysed as both a continuous and categorical variable by sub-division into quartiles. Pearsons correlations were performed to establish any relationships between the various parameters. Comparison between groups of continuous variables was achieved using the Mann-Whitney or Wilcoxon Signed Rank test and categorical variables by chi-square analysis. TL was corrected for age and sex using analysis of covariance analysis. All analyses were performed using SPSS version 15 (SPSS Inc, Chicago, Illinois).

2.4 Sirtuin Relative Expression in Colorectal Cancer Tumour & Normal Tissue

2.4.1 Tissue Processing & RNA Extraction

Specimens were crushed using a mortar and pestle under liquid nitrogen. The resulting tissue powder was then used for RNA extraction which was performed using TRIzol® (Life Technologies) following the manufacturers guidelines; briefly, 50mg of tissue was homogenised in 1ml of TRIzol then incubated for 5 minutes at room temperature. Phase separation was achieved using 200µl of chloroform per 1ml of TRIzol and incubation for 2-3 minutes at room temperature. Samples were then centrifuged (12000g) for 15 minutes at 4^{o}C. The resulting RNA containing aqueous phase was transferred to a fresh tube and the RNA precipitated with 500µl of isopropanol. The samples were then rested for 15 minutes at room temperature and subsequently centrifuged for 10 minutes at 4^{o}C and 12000g. The supernatant was then removed and the RNA pellet suspended in 1ml of 75% ethanol, this was then further centrifuged for 5 minutes at 4^{0}C and 7500g. The supernatant was removed and the resulting RNA pellet air dried. The RNA pellet was dissolved in 30-100µl of RNA/DNA free H_20 and incubated for 10 minutes in a heatblock at 57^{o}C. The RNA concentration and purity of the resulting solution were estimated using the Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, USA). Validation of the RNA was achieved by 1% TBE containing agarose gel electrophoresis. Ethidium bromide 0.5µg/ml and a UV lightbox were used to achieve visualisation (Figure 2.7).

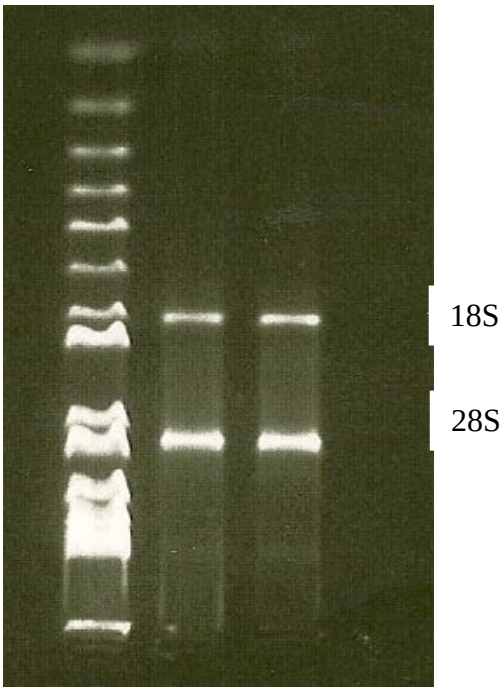

Figure 2.7: Extracted RNA (500ng) from tumour and adjacent colorectal cancer sample run on 1% agarose gel (100V for 90 minutes). Prominent and intact 18S and 28S bands represent ribosomal RNA the presence of which validates the RNA extraction process and sample integrity.

2.4.2 DNAse Treatment

All traces of contaminating DNA was removed from the RNA samples using a commercially available DNA-Free Kit (Ambion, UK) performed following the manufacturer's instructions.

2.4.3 cDNA Synthesis

cDNA for use in the q-PCR assay was synthesised from the extracted RNA by reverse transcription using 2µg of DNase treated RNA. The Superscript First-strand Synthesis System for reverse transcriptase PCR (Invitrogen, UK) was utilised to achieve full length cDNA. Briefly, an RNA/primer mix containing hexamer 50ng/µl, 10mM dNTP, RNA 2µg was made up to a total volume of 12µl with H_2O. This was then incubated at 65^{o}C for 5 minutes followed by 1 minute on ice. 5X first strand buffer, 0.1M DTT and 1µl RNAase out were then added and incubated at 25^{o}C for 2 minutes. The SuperScript II reverse

transcriptase enzyme was then added and incubated at 25^{o}C for 10 minutes, 42^{o}C for 50 minutes and the reaction finally terminated at 75^{o}C for 15 minutes. A parallel reaction without the reverse transcriptase enzyme was used as a negative control. The reaction was completed with RNase H (1µl) and incubated at 37^{o}C for 20 minutes. These samples were then stored at -20^{o}C if not used immediately.

2.4.3.1 Validation of the cDNA Product

A validation reaction was performed to ensure cDNA of sufficient quality had been synthesised during the previous step. This was achieved using a non-quantitative reverse transcriptase PCR reaction using the β-actin housekeeping gene (Appendix 1), this step was performed on both the tissue sample under investigation and corresponding negative control. The reaction mixture was composed of:

- cDNA (12.5ng), forward and reverse primers (400nm), magnesium poor 10X PCR buffer, 10mM dNTP, 50mM $MgCl_2$, Taq DNA polymerase (5U/µl) and nuclease free water to a total volume of 50µl.

The reaction was run using the DNA Engine thermocycler PTC-200 (BioRad) under the following conditions:

- 94^{o}C for 3mins, followed by 30 cycles of 94^{o}C for 45 seconds (denaturing), 61^{o}C for 30 seconds (annealing), 72^{o}C for 1 minute 30 seconds (extension) and a final extension step of 10 minutes at 72^{o}C.

The resulting reaction products were then analysed using 1% agarose gel electrophoresis with samples taken on for use in the RT-PCR step if they displayed the β-actin band at 350bp and the corresponding negative control did not show any amplification which would otherwise indicate contamination of the samples (Figure 2.8).

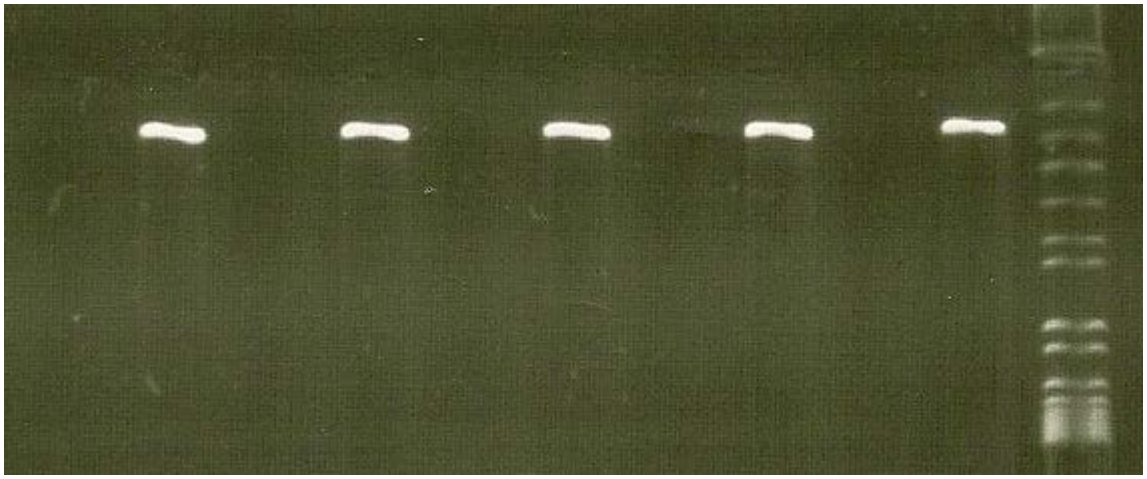

Figure 2.8: 1% TBE agarose gel showing the product of the cDNA synthesis step and subsequent validation by β-actin reverse transcriptase PCR. Each lane represents a tissue sample with corresponding negative control.

2.4.4 Real-time polymerase chain reaction (PCR)

Quantitative RT-PCR was performed to estimate the mRNA expression patterns for SIRT1-7 using the Applied Biosystems 7500 Fast RealTime PCR System (Life Technologies Corporation, CA).

2.4.4.1 Endogenous Reference Gene & Quantification of Gene Expression

Quantification of the sirtuin relative expression in the tumour and normal samples was performed using the $2^{-\Delta\Delta Ct}$ method, otherwise known as the comparative Ct method (as opposed to the standard curve method used for TL determination). This uses an endogenous reference or housekeeping gene in comparison with the target gene, with a final comparison with a normal sample or no template control. In this study the enzyme hypoxanthine ribosyltransferase (HPRT) was used as the endogenous reference gene. This gene had been validated as the reference gene in previous work from our laboratory. Briefly, to be utilised in the experiment the amplification efficiencies between the housekeeping gene and target gene must be approximately equal. This is investigated experimentally by serially diluting the target sample and recording the ΔCt_{sample}, the ΔCt is plotted against the log of the template concentration with a slope of 0 indicating equal efficiency (Zino 2010). HPRT has been further validated in an independent study aiming to

determine the best candidate for use as the endogenous control in RT-PCR experiments using CRC tissue (de Kok *et al*, 2005).

2.4.4.2 Taqman Primer & Probe Validation

As mentioned in the description of the main steps involved in the real-time PCR reaction, a specific TaqMan® primer-probe set which contain the conjugated quencher and reporter tags are required to allow fluorescence detection and subsequent quantification. The sequence of each of the seven SIRT gene forward primer, reverse primer and TaqMan® probe are outlined in Appendix 1. The SIRT4, SIRT5 and SIRT6 primer-probe sets used in the RT-PCR experiment were pre-designed and validated by the company of purchase (Applied Biosystems, UK. Manufacturers code- SIRT4 HS00202033_m1, SIRT5 HS00229729_m1 & SIRT6 HS00213036_m1). The SIRT1, SIRT2, SIRT3. SIRT7 and HPRT primer-probe sets were designed and validated by Mr Samer Zino during the course of previous sirtuin research performed in the host laboratory. These were designed using the Primer Express software programme (Applied Biosystems, UK). From these experiments concentrations of 900nM forward and 900nM reverse were deemed optimal for SIRT1, SIRT2, SIRT3 and HPRT with 300nM forward and 900nM reverse for SIRT7. A probe concentration of 225nM was the most efficient for all five genes (Zino 2010).

2.4.4.3 Reaction Conditions

Real time PCR was performed in 96-well plates using a 25µl reaction. An individual master mix solution was prepared for each gene and to reduce error samples were analysed in batches (Table 2.4).

Table 2.4: Table displaying the constituents for the individual master mix used for each sirtuin and endogenous reference gene analysis experiment.

Master mix for SIRT1-SIRT3, SIRT7 and HPRT	Volume (µl)	Master mix for SIRT4, SIRT5 & SIRT6	Volume (µl)
ABI 2X TaqMan® master mix	12.5	ABI 2X TaqMan® master mix	12.5
Forward primer	1.5	Primer mix	1.25
Reverse primer	1.5	Template (6.25ng/µl)	8
Probe	1	PCR grade water	3.25
Template (6.25ng/µl)	8		
PCR grade water	0.5		
Total	25	Total	25

Primer-probe concentrations for SIRT1-3, SIRT7 and HPRT used were as outlined above. The volume of pre-manufactured primer mix (SIRT4-6) was used to give a final concentration of forward 900nM and reverse 900nM.

Cycle conditions for real-time quantitative PCR were 50^{o}C for 2min, 95^{o}C for 10min followed by 50 cycles of 95^{o}C for 15s and 60^{o}C for 1min.

Configuration of the software on the ABI Fast 7500 thermocycler allowed relative quantification calculations to be performed for each gene in comparison with the HPRT endogenous reference with a normal sample as control. No template and no amplitude controls were also included in batch sample runs for validation purposes. Relative quantification values were the taken forward for analyses with patient variables.

2.4.5 Statistical Analysis

Relative expression levels are expressed as median and IQR or 95% confidence interval where appropriate. Differences in expression between the tissue groups were determined using the Mann-Whitney or Wilcoxon Signed Rank test where appropriate. Sirtuin expression levels were correlated with clinico-pathological characteristics as a continuous variable using Pearsons correlation analysis and categorical variable using chi-square, Mann-Whitney or Kruskal-Wallis where appropriate. Bonferroni correction was used in the analysis of inter-relationships between individual sirtuins. Determination of a relationship with survival was performed using Kaplan-Meier and log rank analysis. All analyses were performed using SPSS version 15.0 (Chicago, Illinois, USA).

CHAPTER 3: TELOMERE LENGTH AND COLORECTAL CANCER

3.1 Introduction

Colorectal cancer (CRC) is the third most common cancer in the UK and is responsible for approximately 16,000 deaths every year. Increasing chronological age is a risk factor for many types of cancer including colorectal, with eighty percent of CRC cases occurring in patients over the age of sixty (Cancer Research UK 2010). Consequently, an understanding of the biology of ageing may provide insight into cancer pathogenesis (Lamb & Shiels 2009). Biological ageing comprises ageing at the cellular and organ level and is affected by genetic, metabolic and environmental factors. Fully delineating the key molecular mechanisms underpinning both the biological ageing and cancer processes could improve the understanding of the disease process and lead to the discovery of novel biomarkers or targets for therapeutic intervention, further improving survival rates.

A key manifestation of ageing at the cellular level is telomere attrition. Telomeres are nucleoprotein structures located at the ends of all eukaryotic chromosomes and are composed of a repetitive guanine-rich DNA sequence $(TTAGGG)_n$ (Moyzis *et al,* 1988). They possess a number of critical functions including maintenance of genomic integrity by protecting chromosomes from fusion events, repair of DNA damage and maintenance of cellular stability (Lamb & Shiels 2009). Telomere attrition is associated with increasing chronological age and furthermore may act as a biomarker of replicative ageing, or mitotic clock (Harley *et al,* 1990). Once this progressive loss of telomeric DNA content reaches a critical level, cells are stimulated to either apoptose, or enter replicative senescence (Hayflick & Moorhead 1961).

Evidence is accumulating that telomere attrition and senescence are contributory factors in a range of age related disease processes including cancer (Blasco 2005), cardiovascular disease (Brouilette *et al,* 2007), chronic kidney disease (Carrero *et al,* 2008) and pulmonary disease (Savale *et al,* 2009). Furthermore, critically short telomeres have been linked with life stress (Epel *et al,* 2004, Shiels *et al,* 2011) and an overall increased likelihood of mortality (Cawthon *et al,* 2003). In essence, telomere attrition in PBLs reflects ‘miles on the clock’ and a corresponding increased likelihood of disease and mortality. The body of work aimed at delineating the relationship between critical telomere attrition and the cancer process is rapidly expanding. Short telomeres in peripheral blood leucocytes (PBL) have been shown to be associated with an increased risk in a variety of

solid tumours (Wu *et al*, 2003, Jang *et al*, 2008, Hou *et al*, 2009, Mirabello *et al*, 2010). However, results of recent studies from the same laboratory have indicated an increased risk of breast and renal cell carcinoma with increased and not decreased TL (Svenson *et al*, 2008, Svenson *et al*, 2009, Svenson& Roos 2009). These results are counter-intuitive and are divergent from the majority of pertinent work in the area.

Chapter Aims

- To test the hypothesis that patients with colorectal cancer display evidence of accelerated biological ageing in the form of telomere attrition when compared with healthy controls.

- By determining telomere length in PBL, tumour tissue and normal colonic tissue we aim to provide key information on telomere dynamics in each of these important cell compartments.

- Correlate PBL and tissue telomere length with clinical and pathological outcomes to determine whether telomere length could prove useful as a prognostic tool.

3.2 Results

3.2.1 Analysis of telomere lengths in PBLs of CRC patients and healthy controls

Sixty-four (64) patients were available for analysis in the CRC PBL patient group (mean age = 68 ± 10.8). These were compared with one thousand three hundred and forty eight (1348) West of Scotland control subjects (no diagnosed cancer), aged approximately 57 (n=847, male 46%, female 54%) and 76 (n=501, male 42%, female 58%) years old (mean age = 64 ± 9.24). As expected there was a significant negative association between chronological age and TL in the healthy controls (Pearson r=-0.215, $p<0.001$). Likewise a significant negative relationship was observed between chronological age and PBL telomere length in the CRC group (Pearson r=-0.257, p=0.04, Figure 3.1A), indicating that as patient age increased, TL decreased. Although this negative association was maintained when the CRC PBL group was split according to sex (Figure 3.1B), the relationship did not

reach significance in either males (Pearson r=-2.43, p=0.123) or females (Pearson r=-2.47, p=0.187). This age-related telomere attrition demonstrates the association between chronological and biological ageing.

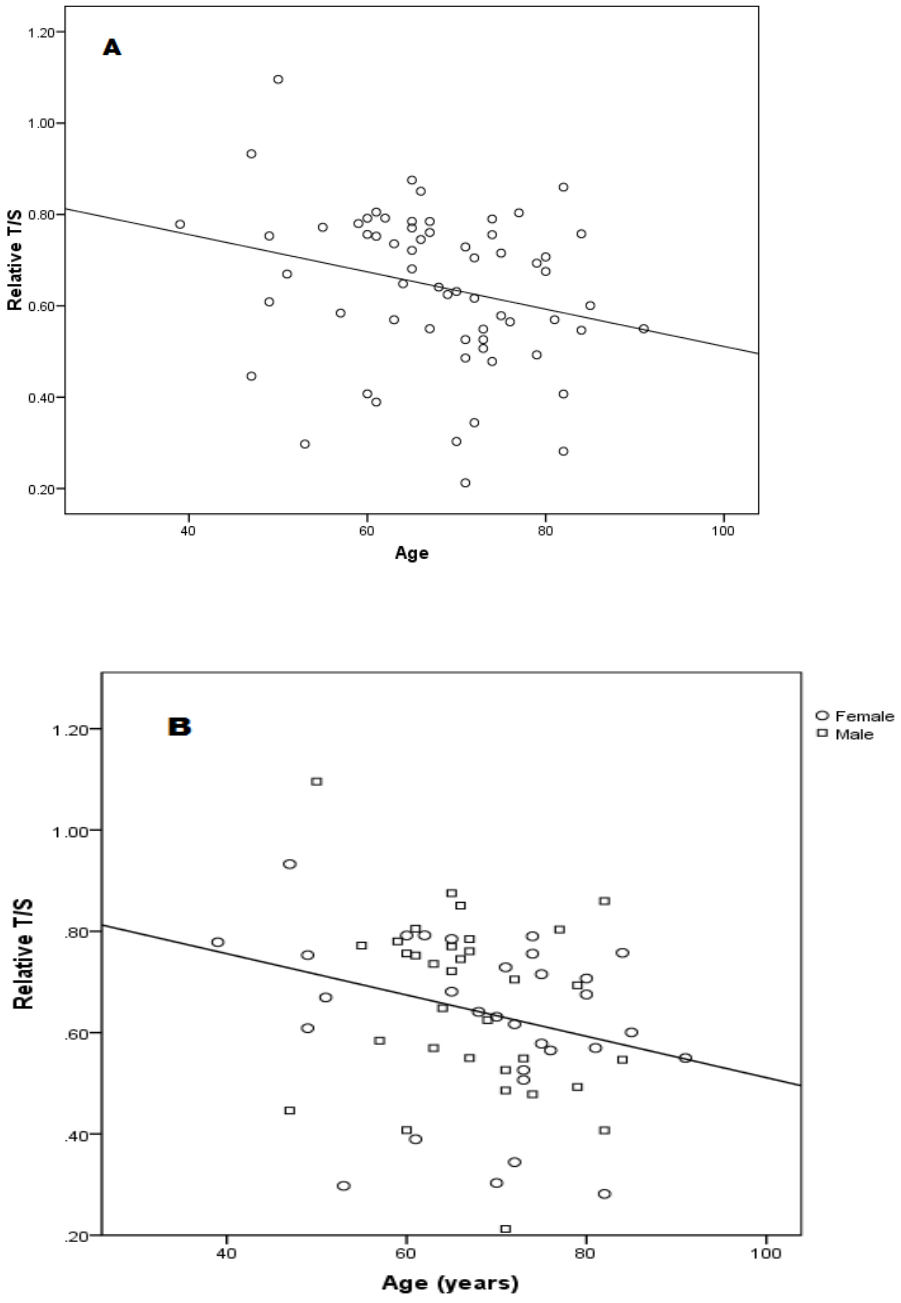

Figure 3.1: (A) Scatter plot displaying the significant negative correlation between relative T/S and chronological age in the CRC PBL group (Pearson r=-0.257, p=0.04). (B) Similar scatter plot with CRC PBL group split according to sex, relationship not significant. Trend line identical for male and female.

Patients had consistently shorter telomeres than the control population ($p<0.001$) (Figure 3.2), indicating that the CRC patients were of increased biological age. Since the median age of the control population was greater than that of the CRC group, analyses were performed correcting for both age and gender. After correction of TL for age and gender, colorectal cancer patients still had consistently shorter telomeres (adjusted mean RelT/S=0.66±0.02(se)) compared with those in the control group (adjusted mean RelT/S=0.75±0.005(se), $p<0.001$), indicating that the colorectal cancer patients were of increased biological age. To further validate that the cancer patients were more biologically aged, we compared them to a sub-group of the control population, those individuals aged 76yrs. Analysis of covariance revealed that despite being on average chronologically younger, the cancer group had significantly shorter telomeres (adjusted mean RelT/S=0.61±0.03(se)) than the control population (adjusted mean RelT/S=0.70±0.01(se)) and hence were more biologically aged ($p=0.004$). The median duration of diagnosis in the CRC PBL group (defined as the date of positive tissue diagnosis until date of sample collection) was 42 (6-185) days. Analysis did not reveal any association between duration of diagnosis and TL.

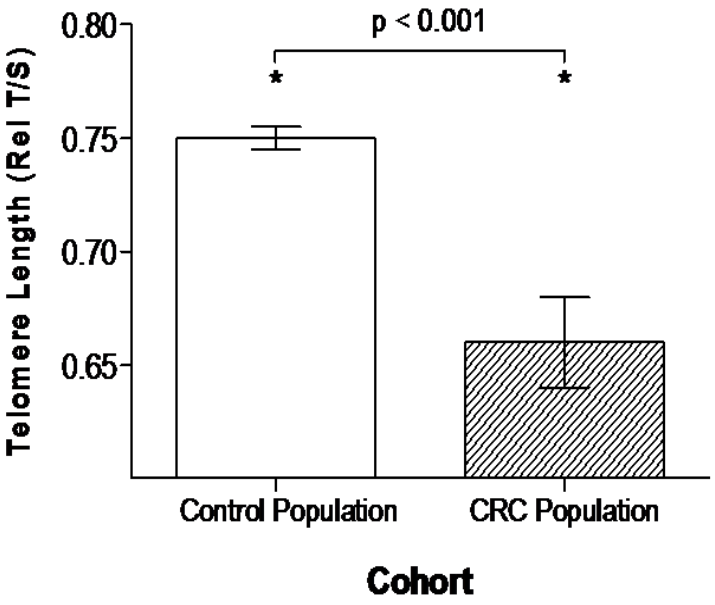

Figure 3.2: Measurement of PBL telomeres from a control and colorectal cancer (CRC) population revealed that the CRC patients had significantly shorter telomeres (Mean telomere length ± standard error (Rel T/S 0.66 ± 0.02) than the control population ((0.75 ± 0.05) $p<0.001$).

3.2.2 Clinico-pathological Correlation with CRC PBL Telomere Length

No associations, other than chronological age were observed between PBL telomere length and the various clinico-pathological parameters when analysed using the continuous variable. Consequently, patients were categorised into those with short telomeres (Relative T/S < 0.55, shortest quartile) and those with long telomeres (Relative T/S > 0.55). Short TL was significantly positively associated with high risk pathological features indicated by a high risk Peterson Index (chi square, P=0.035). However, no significant relationship was identified between tumour site, Dukes stage, and any other clinico-pathological characteristic measured.

3.1.2.3 Socio-Economic Correlation with Telomere Length

The Carstairs index was used to stratify the patients in the CRC PBL group according to levels of socio-economic deprivation. This system uses four variables; overcrowding, male unemployment, low social class and lack of motor vehicle to compose an overall score for a postcode sector. Every postcode sector in Scotland is therefore assigned a score ranging from 1 (least deprived) to 7 (most deprived) (Carstairs& Morris 1989). The distribution of Carstairs index scores in this group of patients is outlined in (Table 2.1). There is a clear preponderance of patients from areas of socio-economic deprivation with 43.75% residing in areas with a Carstairs Index of 7. There was no apparent significant relationship demonstrated between levels of socio-economic deprivation and TL, in this group of patients (Figure 3.3). However, this study is not correctly designed or powered to tease out relationships between TL and population variables such as deprivation, this will be addressed in due course.

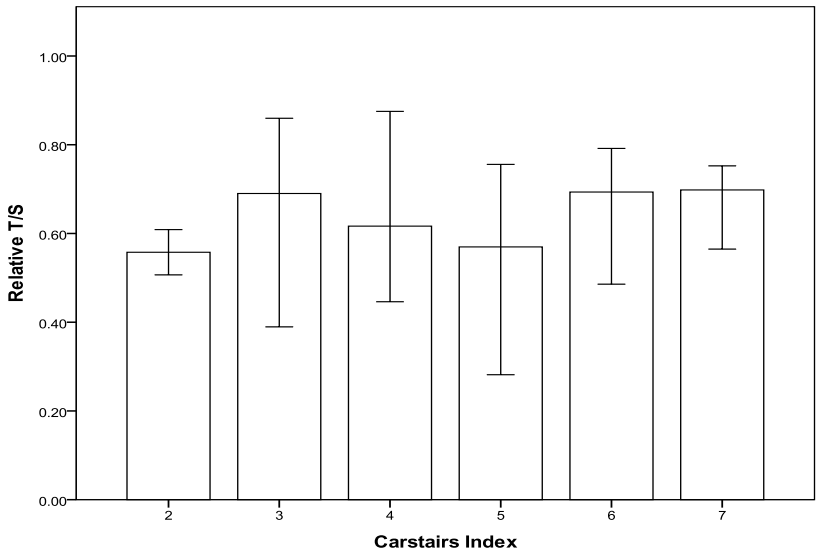

Figure 3.3: Bar chart displaying telomere length stratified according to the Carstairs Index of deprivation. There was no significant relationship between telomere length and the different levels of deprivation score (error bars ± 95% confidence interval)

3.1.2.4 Comparison of telomere length in colorectal tumour tissue and normal adjacent tissue

The relative T/S ratio of 32 matched colorectal tumour tissue and adjacent normal mucosa samples was compared to determine whether a difference in biological age was apparent between tissue types. Telomere length in the tumour tissue (median=0.43, IQR=0.40) was found to be significantly shorter than in the adjacent normal tissue (median=0.65, IQR=0.28) (p=0.004, Figure 3.4). Additionally, a comparison was made between the TL from the three tissue sources. There was no significant difference in TL between the CRC PBL group and that from the normal CRC tissue group (Figure 3.5).

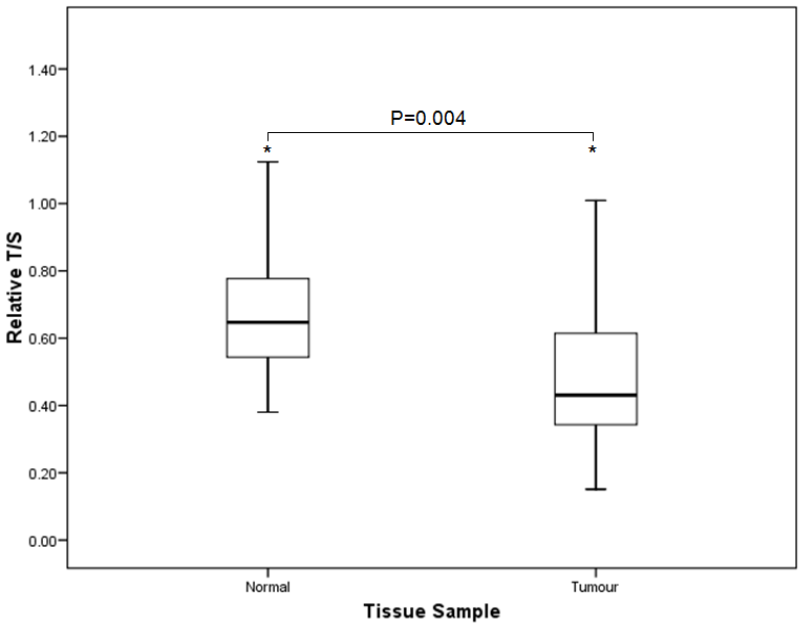

Figure 3.4: Box plot highlighting the difference in telomere lengths between thirty two matched tumour and adjacent normal tissue samples of colorectal cancer patients. Tumour tissue (median Rel T/S = 0.43) displayed significantly shorter telomeres than normal tissue samples (median Rel T/S = 0.65, Wilcoxon signed rank test, p=0.004).

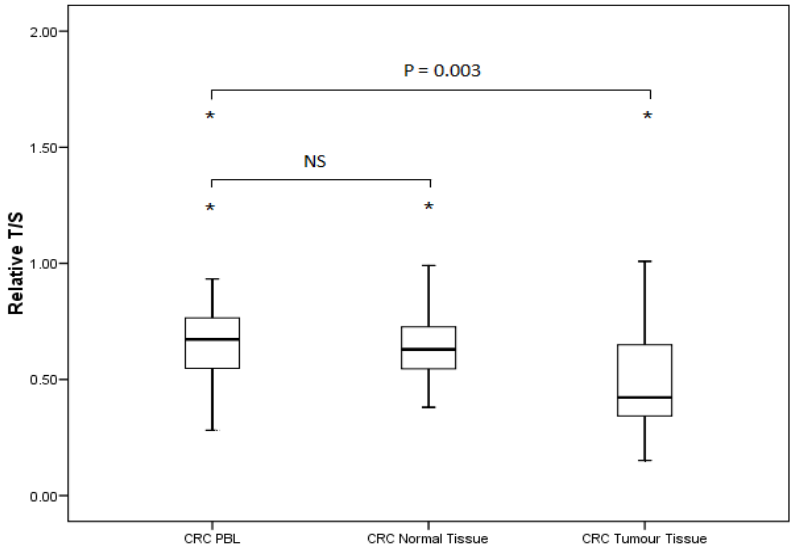

Figure 3.5: Box plot displaying the difference between the difference telomere length between the PBL and tissue group. No significant difference between the TL in PBL and normal tissue. TL in CRC tumour tissue significantly shorter than TL in CRC PBL (Mann-Whitney, p=0.003).

3.1.2.5 Clinico-pathological Correlation with Tissue Telomere Length

Analysis of TL from both CRC tissue and normal adjacent mucosa as a continuous or categorical variable did not reveal any significant associations with the clinic-pathological variables outlined in Table 2.1. Of particular note and in contrary to the relationship seen in the CRC PBL group there was no association between CRC tissue TL with chronological age, nor any sex difference. Although there was no significant association with any pathological variables there was an interesting trend in TL with Dukes stage where TL decreased progressively from Dukes' A to C then increased in Dukes D patients (Figure 3.6A). There was no significant difference between CRC tissue TL according to tumour site, with median rectal relative T/S 0.33 (±0.79) compared with colonic relative T/S 0.42 (±0.93), $p = 0.412$ (Figure 3.6B).

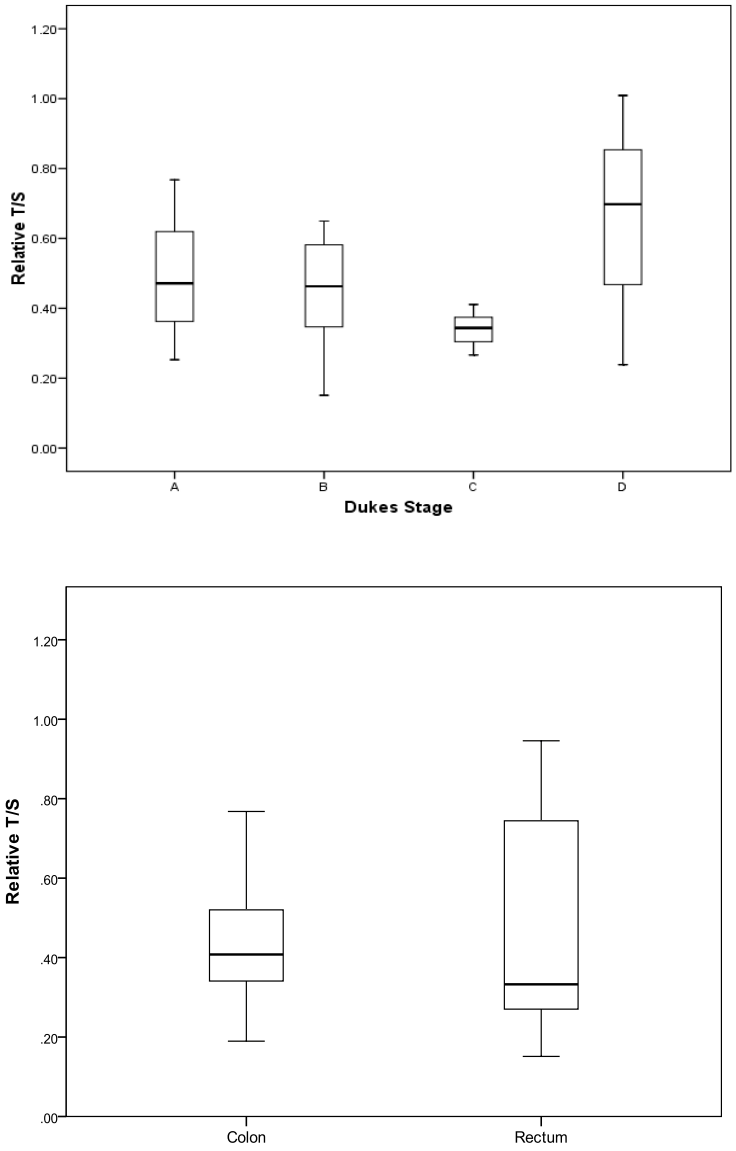

Figure 3.6: (A) Box plot displaying TL in CRC tissue samples in relation to Dukes stage. No significant relationship demonstrated. Dukes A- Tumour limited to muscularis propria, Dukes B- local spread beyond muscularis propria, Dukes C- nodal spread, Dukes D- Distant metastasis (B) Box plot displaying non-significant difference between colonic and rectal tissue telomere length (p=.412)

3.3 Discussion

3.3.1 Patients with CRC display evidence of accelerated biological ageing

This study demonstrates that patients with colorectal cancer display clear evidence of accelerated biological ageing in the form of telomere attrition when compared with healthy

control subjects (Maxwell *et al,* 2011). The relationship between TL and CRC risk is proving to be a difficult one to fully delineate but highlights the inter-individual variation in both biological ageing and the effect of potential confounders. Demonstration of telomere attrition in the PBLs of CRC cancer patients is similar to that reported by Pooley et al (2010) in both retro- and prospectively recruited patients (Pooley *et al,* 2010). These data contradict two studies of both male and female CRC patients where no relationship between CRC and TL was identified (Lee *et al,* 2010, Zee *et al,* 2009).

The intricacies of the relationship between TL and CRC are highlighted in a recent study by Jones *et al* (2011). The authors report a significant association between a SNP at the *TERC* locus, long telomeres and increased CRC risk. The supposition from this data is that longer telomeres predispose to CRC cancer, potentially resulting from an increased replicative capacity of cells with longer telomeres and hence more chance of acquiring tumour-causing mutations. However, in the same study the authors report that TL in PBLs from CRC patients is shorter than control subjects. This relationship was determined using DNA from patients enrolled in three different trials involving varying aspects of CRC management. The authors use these differences in populations as an explanation of the seemingly paradoxical results within this study (Jones *et al,* 2011). Prevalent studies investigating a number of other cancer entities including gastric (Hou *et al,* 2009), bladder (Wu *et al,* 2003), ovarian (Mirabello *et al,* 2010) and lung (Jang *et al,* 2008) are, however, in concordance with our observations.

Clearly some heterogeneity exists between studies attempting to determine the relationship between TL and varying types of cancer. In order to eliminate some of this heterogeneity between studies and increase the sample size under investigation Ma *et al* (2011) have recently reported a meta-analysis, which provides perhaps the most compelling evidence for an association between cancer risk and shortened PBL TL. The authors amalgamated previously published data thereby producing a study population for investigation which contained 11,255 cases and 13,101 controls. They report that shorter telomeres were significantly associated with some individual cancers for example bladder and lung, and also grouped cancers such as those of the gastrointestinal tract and urogenital system (Ma *et al,* 2011). Another recent meta-analysis has confirmed this association of short TL with overall cancer risk in retrospective analyses (Wentzensen *et al,* 2011). Prospective as opposed to retrospective studies have the advantage that they are less open to observer bias and other potentially confounding factors. The results of these studies are again

heterogeneous. Most recently Willeit *et al* (2011) have reported long-term follow up of a random sample of 1000 persons aged 40-79 years. TL was measured at two time points and cancer incidence recorded over a 15 year period. The authors report a change in TL over time with short telomeres associated with an increased cancer risk and mortality from cancer. This study corroborates previously reported 10 year follow up data from the same authors (Willeit *et al,* 2010, Willeit *et al,* 2011). However, meta-analysis of prospective studies has not revealed any significant association between short TL and over-all cancer risk (Wentzensen *et al,* 2011). Analysis of prospective longitudinal studies must be done with caution as they, depending on the design, cannot look at the relative rate of change compared with a baseline (birth) TL. Thus any change may be a regression to the mean and not a true reflection of TL change over a lifetime.

The original hypothesis of this study was to determine if patients with CRC display evidence of accelerated biological ageing. The significant difference in TL between the PBL CRC and control group indicates advanced biological age in the CRC group and supports the original hypothesis. This is exemplified by the fact that when the analyses were corrected for age the significant difference in TL remained, meaning that although the two groups were similar in chronological age the cancer patients displayed evidence of accelerated biological ageing. The possibility exists that accelerated ageing arises as a consequence of the disease process (Svenson & Roos 2009). Possible reasons for this include the fact that the cancer process is well known to induce a pro-oxidative and systemic inflammatory state (Leung *et al,* 2008). If this were the case then it would be expected that TL would continue to shorten the longer the disease progressed. Longer telomeres closer to diagnosis have been demonstrated in CRC patients previously (Jones *et al,* 2011). However, our analysis refutes this, as there was no relationship between TL and the duration of the disease prior to sample collection. It may be that our median duration of 42 days between diagnosis and sample collection is not sufficient to observe any effect elicited by the disease process. This could only be resolved by making serial measurements of TL throughout the course of the disease, but this is almost certainly an impossible aim as treatment of the tumour either with palliative or curative intent could confound results. It would also be of interest to monitor the effect of curative resection of the tumour, if the disease process is the driver of telomere attrition then this should be attenuated when the tumour is removed.

Although analysis did not reveal any association between socio-economic deprivation and TL in our group, recent evidence has suggested such a link exists in a population with similar demographics and spectrum of socio-economic deprivation (Shiels *et al,* 2011). In this study lower socio-economic class and poor diet contributed to accelerated biological ageing, represented by telomere attrition. Furthermore, there was a potential association with the pro-inflammatory cytokine IL-6. This study provides evidence supporting the link between social deprivation and a biological explanation of the excess disease burden, including cancer seen in this population. It is likely that the number of participants in the current study prohibited determination of any association between socio-economic deprivation and altered telomere dynamics.

Our analysis suggests that the cancer patients with the shortest PBL telomeres have pathologically more severe disease as indicated by a higher Peterson Index. This is an intriguing observation as it indicates that systemic telomere biology could not only affect the risk of CRC but also the pathological outcome. It is, therefore relevant that there is no significant difference in TL between PBL and normal colonic tissue comparing our two sample groups (Figure 3.2). It should be borne in mind that this comparison is not direct i.e. the blood sample and colonic tissue sample were not from the same patient. The groups are, however, comparable in terms of demographics and pathology. No study has ever reported on the compartmental telomere dynamics in cancer patients. Clearly, direct comparison would need to be made before firm conclusions could be drawn but PBL TL could prove useful as a surrogate marker of pathological aggressiveness and could allow risk stratification prior to commencement of specific treatment algorithms.

As outlined in the introduction to this thesis telomeres have been shown to be critical mediators of genomic integrity with telomere attrition associated with major chromosomal abnormalities. One of the key concepts in the process of genomic instability induced by dysfunctional telomeres is that of crisis. This is a period of rampant genomic instability and cell death which is driven by continued telomere erosion and uncapping in cells deficient in p53/pRB. Mutation of p53 is critical, as up-regulation of wild type p53 would in normal circumstances facilitate senescence and prevent ongoing proliferation. It is therefore pertinent that 50-75% of colorectal cancers express some form of deleterious mutation of p53 (Pino & Chung 2010). Clearly this state of crisis is not compatible with the enhanced proliferative capacity of neoplastic cells. However, neoplastic cells almost

universally adopt telomere maintenance programmes (either re-activation of telomerase or ALT) which stabilise telomeres and allow continuing replication. Replication proceeds in the context of a permissive mutated genome which is reflected in the chromosomal aberrations typical of many human cancers including colorectal (Counter *et al,* 1992, Raynaud *et al,* 2008, Deng *et al,* 2008). This permissive mutated genome could explain the step-wise mutational model of colorectal carcinogenesis proposed by (Fearon& Vogelstein 1990). Whereby, the process of carcinogenesis is initiated by mutation of the APC tumour suppressor gene, followed by activating mutation of KRAS and subsequent mutation of p53, TGF-β and PIK3CA. It is this sequence of mutations which drives the adenoma-carcinoma sequence of events in colorectal carcinogenesis (Leslie *et al,* 2002).

3.3.2 Tissue telomere dynamics in colorectal cancer

In concordance with previous studies we have confirmed that TL in CRC tissue is significantly shorter than normal adjacent colorectal mucosa (Hastie *et al,* 1990, Rosenberg *et al,* 2003, Gertler *et al,* 2004, Rampazzo *et al,* 2010). One might expect that given telomerase activity in neoplastic cells, including those of a colorectal origin, TL would be elongated in representative neoplastic tissue. However, our data suggest the opposite, meaning telomerase must maintain telomeric DNA content at a level consistent with a high rate of cell proliferation. This avoids the initiation of senescence or apoptosis which would otherwise mean exit from the cell cycle, and prohibit the rapid proliferation of neoplastic cells. These cells can therefore continue to divide but do so with requisite maintenance of TL. Telomerase is reactivated in colorectal cancer. Activation has also been shown to reflect progression through the adenoma-carcinoma sequence. Yan *et al* (1999) have demonstrated that telomerase activity was significantly associated with the progression of adenomatous polyps from low to high grade, with activation universally demonstrated in all carcinoma specimens investigated (Yan *et al,* 1999). Studies have attempted to correlate TL with the various stages of adenoma-carcinoma sequence of CRC development. TL in epithelial cells at the earliest morphological definable stage of carcinoma (high grade dysplasia with minimal invasive growth) was shorter compared with surrounding adenoma, indicating that cancers arise from cells with critically short telomeres within the adenoma (Plentz *et al,* 2003).

In this study there was no significant correlation of CRC tissue TL with any of the clinico-pathological parameters in Table 2.1. However, as shown in Figure 3.6 TL appears to be longer in patients with more advanced disease (Dukes D). Although not statistically

significant this is pertinent as longer TL in more advanced disease is a recurring finding in analysis of tissue specimens of CRC. Both Gertler *et al* (2004) and Garcia-Aranda *et al* (2006) report that tissue TL is shorter in cancer specimens when compared with adjacent normal tissue. They also both report a significant association between longer carcinoma TL, more advanced disease (Dukes C and D) and poor prognosis (Gertler *et al*, 2004, Garcia-Aranda *et al*, 2006). Increased telomerase expression in CRC tissue samples has also been shown to correlate with poorer prognosis (Tatsumoto *et al*, 2000) and more aggressive disease (Simsek *et al*, 2010). It is therefore postulated that telomere attrition and subsequent chromosomal instability is required for the progression from dysplasia to neoplasia. But, upregulation of telomere maintenance mechanisms such as telomerase confers a proliferative advantage which is reflected in the association between longer TL, high telomerase expression levels and poor prognosis in CRC. Whilst we noted similar findings to those previously reported these were not significant and it may be that increasing the power of our study could replicate these significant recurring findings of increased TL with more advanced disease.

As expected we have demonstrated that patients with CRC display evidence of accelerated biological ageing and that telomere dynamics in different tissue compartments in these patients are altered. As already mentioned the mechanisms controlling TL are only partly understood. We therefore attempted to correlate our TL measurements with factors involved in the control of the redox state and systemic inflammation, both known to impact on TL at a number of levels.

CHAPTER 4: CORRELATES OF TELOMERE LENGTH IN COLORECTAL CANCER

4.1 Introduction

Telomeres not only potentially serve as biomarkers of senescence and biological ageing, but also form part of a damage sensing and signalling system, facilitating DNA repair or apoptosis (Misri *et al,* 2008). Potential determinants of TL are varied. Telomere length is highly heritable therefore a proportion is genetically determined (Slagboom *et al,* 1994). Alteration in the expression of telomerase, can significantly affect telomere dynamics. In mice, knock out of the telomerase coding sequence resulted in progressive loss of telomeric DNA and progeria (Blasco *et al,* 1997). Reintroduction of telomerase reversed both these effects (Samper *et al,* 2001). A major influence on TL and hence telomere function is the control of redox state and potential damage induced by reactive oxygen species (Saretzki & Von Zglinicki 2002, von Zglinicki 2002). Correlation of TL with genes controlling redox state in a narrow age range cohort provides further evidence for a plausible mechanistic link between redox control and telomere biology (Starr *et al,* 2008). Pertinent in this respect, is the observation that fetuin-A, a mediator of redox homeostasis in the circulation, displays a dependent relationship with PBL TL in patients with chronic kidney disease. (Carrero *et al,* 2008). Fetuin-A is a ubiquitous serum protein which plays a role in extra-cellular calcium metabolism by virtue of its ability to inhibit calcium-phosphate precipitation and is an important inhibitor of extra-skeletal calcification, an effect important in patients with chronic kidney disease on dialysis (Westenfeld *et al,* 2007). Furthermore fetuin-levels are down-regulated according to the acute phase inflammatory response in a manner similar to serum albumin, corresponding to an inverse relationship with C-reactive protein (CRP) and inflammatory cytokines (Dervisoglu *et al,* 2008). In clinical terms the effects of fetuin-A are complex with both high and low levels predicting risk and outcome of ischaemic heart disease and stroke. These differences could result from the differing effects of fetuin-A not only on inflammation but also on adhesion molecule interaction (Weikert *et al,* 2008, Bilgir *et al,* 2010).

Other factors known to generate potentially damaging reactive oxygen species includes altered antioxidant ratio, disordered calcium and other divalent cation homeostasis, and alteration of micronutrient status. In addition to damage induced by reactive oxygen

species, inflammation has been shown to play a determining role in experimental and population based models of telomere attrition (Carrero *et al*, 2008, Shiels *et al*, 2011). The overall effect of oxidative damage and systemic inflammation is reflected in the correlation between PBL TL and solid organ biological age. Indeed in their work in vascular disease Wilson *et al* (2008) report that PBL TL is strongly correlated with vascular wall TL, thus indicating that PBL TL acts as a surrogate for vascular wall aging and supporting the notion that factors influencing TL do so at a systemic level (Wilson *et al*, 2008). Furthermore chronic systemic inflammation has a well characterised deleterious role in the development and progression of cancer, and in particular CRC (Roxburgh & McMillan 2010).

Chapter Aims

- To assess whether PBL TL in CRC patients were reflected in markers of systemic inflammation including the mGPS, pro-inflammatory cytokines and common haematological indices of inflammation.

- Delineate the relationship between PBL TL and factors known to exert control over redox state including antioxidant vitamins, factors involved in calcium homeostasis including circulating levels of fetuin-A and micronutrient status.

- Further characterise the interplay between fetuin-A, inflammation and clinical parameters in our CRC patients.

4.2 Results

4.2.1 Correlation of CRC PBL telomere length and redox state

4.1.2.1 *No Association between Telomere Length and Antioxidant Vitamins or trace elements*

There was no significant association between TL as either a continuous or categorical variable, and anti-oxidant status as determined by correlation with serum levels of

antioxidant vitamins (retinol, α-tocopherol, lutein, lycopene, α-carotene and β-carotene) (Table 4.1). In addition, there was no correlation between TL and serum levels of the micronutrient trace elements; Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} and Se^{2+} (Table 4.1). Furthermore, there was no correlation between any of the redox control factors and any clinico-pathological parameters.

Table 4.1: Table displaying relationship between CRC PBL telomere length with antioxidant vitamins and trace elements.

	Antioxidant Vitamins	
	Median (range)	**Correlation with Telomere length‡ (p<0.05)**
Retinol	1.80 (0.7-3.70) µmol/L	1.67 (0.298)
α-tocopherol	26.5 (12.0-40.0) µmol/L	0.072 (0.652)
Lutein	88.0 (21.0-607.0) µg/L	0.047 (0.772)
Lycopene	83.0 (10.0-373.0) µg/L	-0.006 (0.971)
α-Carotene	15.5 (10.0-151.0) µg/L	0.178 (0.260)
β-Carotene	65.5 (10.0-862.0) µg/L	-0.174 (0.271)
	Micronutrient Trace Elements	
Magnesium (mmol/l)	0.82 (0.56-1.11)	0.009 (0.947)
Iron (Fe^{2+}, µmol/l)	20.2 (5.6-168.7)	0.067 (0.631)
Copper (µmol/l)	19.4 (12.7-31.9)	0.132 (0.345)
Zinc (µmol/l)	10.3 (6.6-14.1)	0.05 (0.721)
Selenium (µmol/l)	0.84 (0.45-1.23)	0.143 (0.306)

‡ Relationship displayed as Pearson correlation with telomere length as continuous variable (p<0.05).

4.1.2.2 Relationship between CRC PBL telomere length and calcium homeostasis

An attempt was made to determine any relationship between TL, plasma calcium concentration and factors involved in calcium homeostasis, namely vitamin D. When analysed as both categorical and continuous variables there was no relationship between TL and plasma calcium. Likewise there was no relationship between TL and vitamin D levels (n=26, Pearson r -0.75, p=0.715, Table 4.2).

Table 4.2: Table displaying the relationship between telomere length and elements associated with calcium homeostasis. Inter-relationship between Fetuin-A and elements of systemic inflammation.

Calcium Homeostasis

	Median (range)	Correlation with telomere length (p<0.05)
Fetuin-A	27.16 (14.71-67.27) ng/ml	**0.041**[†]
[*]**Age**	r= -0.32	**0.011**
[*]**Albumin**	r= 0.28	**0.03**
[*]**Calcium**	r= 0.30	**0.022**
[*]**IL-10**	r= -0.21	0.061
[*]**IL-6**	r= -0.483	**0.005**
Calcium	2.44 (2.31-2.61) mmol/L	0.072 (0.590)[‡]
Vitamin D	31.5 (11-111) nmol/L	-0.75 (0.715)[‡]

[†]Patients with low fetuin-A (fetuin-A < log median=1.47/median=29.6) had significantly shorter telomeres (median T/S = 0.6 Vs 0.72, Mann-Whitney).
[*]Relationship with plasma log Fetuin-A concentration, displayed as Pearson correlation.
‡ Relationship displayed as Pearson correlation with telomere length as continuous variable (p<0.05).

4.2.2 Plasma levels of Fetuin-A are associated with chronological and biological age in colorectal cancer

Fetuin-A concentration of plasma samples was measured to assess whether levels were associated with chronological and biological ageing within the CRC PBL group. A significant relationship was observed between fetuin-A concentration and the chronological age of subjects (Pearson r=-0.32, p=0.011, Figure 4.1A, Table 4.2), increasing chronological age was associated with decreasing fetuin-A concentration. There was no significant association between TL and fetuin-A. Therefore, further analysis of the relationship between fetuin-A concentration and TL was performed. Patients were categorised into two groups around the median plasma level giving a low fetuin-A level group (fetuin-A < log median=1.47/median=29.6) and a high fetuin-A level group (fetuin-A > log median=1.47/median=29.6). Patients with low fetuin-A levels were shown to have significantly shorter telomeres (median RelT/S = 0.6) than those patients with high fetuin-A levels (median RelT/S = 0.72) (Pearson r=0.3, p=0.019, Mann Whitney p=0.041, Table 4.2), this relationship was maintained when the analysis was adjusted for age. Patients with low fetuin-A levels had significantly shorter telomeres (adjusted mean Rel T/S=0.59) in comparison to those patients with high fetuin-A levels (adjusted mean RelT/S =0.68,

p=0.047, Figure 4.1B). No difference in fetuin-A level was observed between males and females.

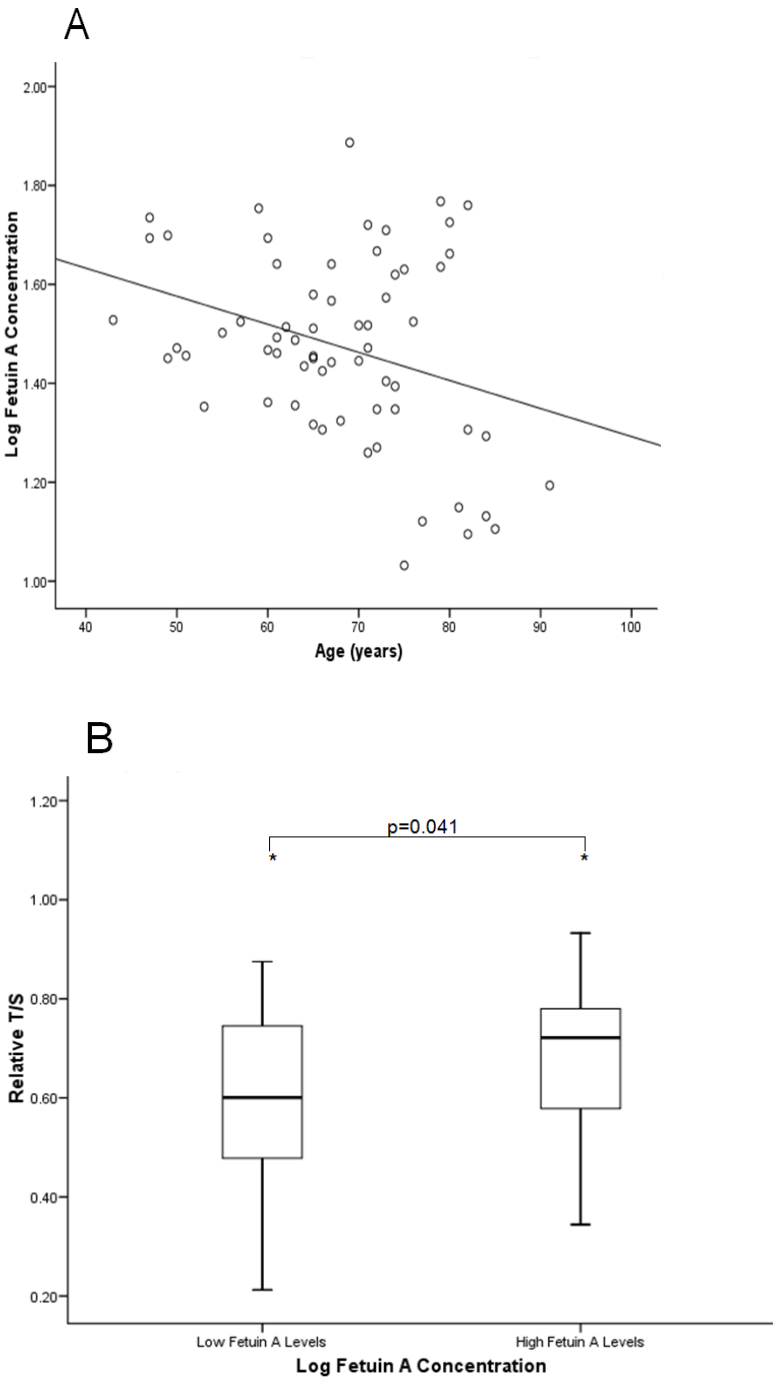

Figure 4.1: Scatterplot (A) illustrating the significant negative correlation between patient chronological age and plasma fetuin-A level (pearson = -0.32, p=0.011). Boxplot (B) displaying the association between plasma fetuin-A level and PBL telomere length in colorectal cancer patients. Patients with low levels of fetuin-A had significantly shorter telomeres when compared with those with higher Fetuin-A levels (Mann-Whitney, p=0.041).

4.2.2.1 Association between plasma Fetuin-A levels and patient clinico-pathological parameters

No association was apparent between fetuin-A concentration and tumour characteristics such as T-stage, lymph node involvement or Dukes stage, within the CRC PBL group. However, increasing concentrations of fetuin-A were significantly associated with increasing levels of albumin (Pearson r=0.28, p = 0.03) and calcium (Pearson r=0.30, p=0.022), but decreasing levels of IL-6 (Pearson r=-0.483, p=0.005) (Table 4.2, Figure 4.2). A trend also existed between fetuin-A concentration and IL-10 (Pearson r=-0.21, p=0.061) (Table 4.2, Figure 4.2), whereby increasing concentrations of fetuin-A in patients was associated with decreasing levels of IL-10 (Figure 4.2).

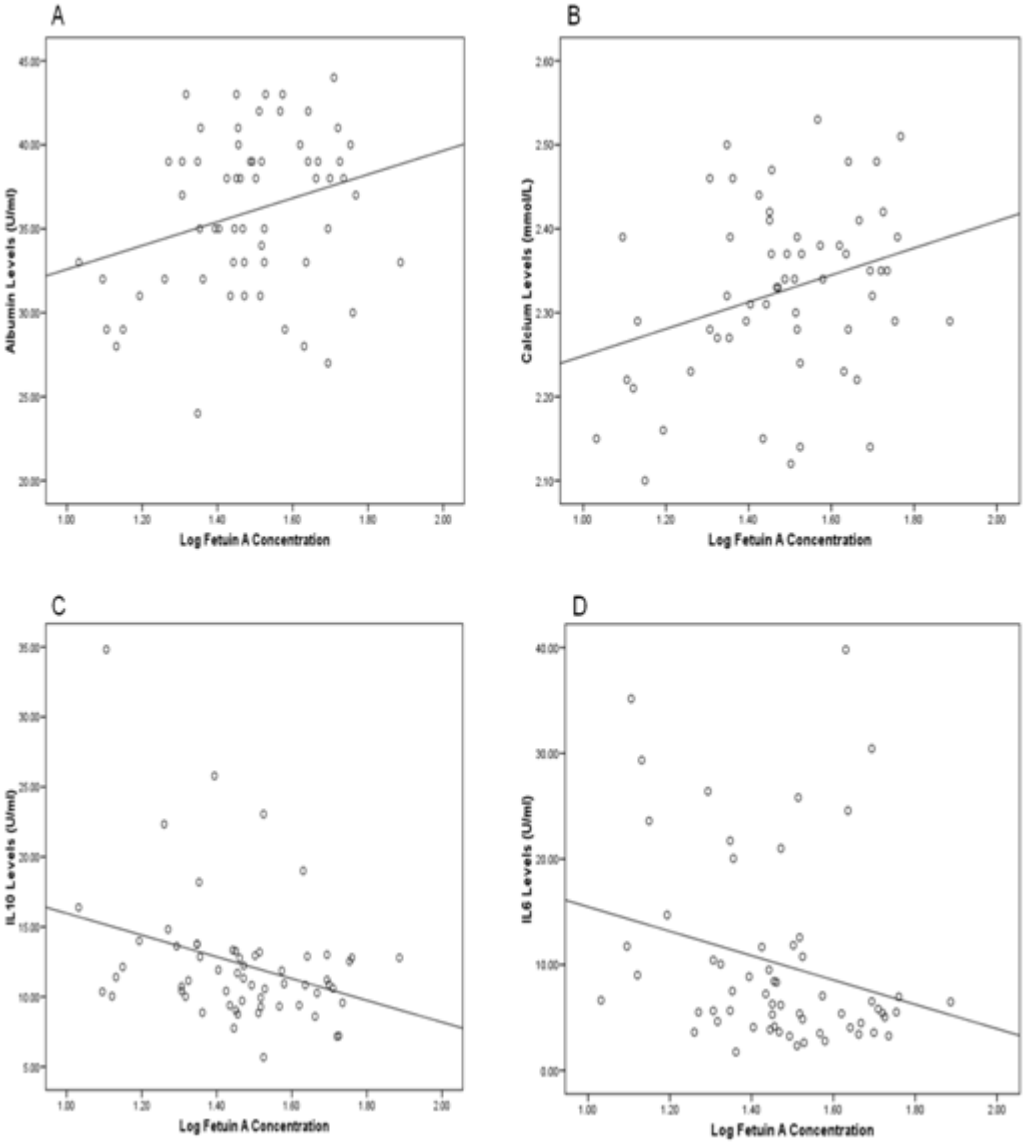

Figure 4.2: Scatter plot displaying the relationship between albumin (A), calcium (B), interleukin-10 (C), interleukin-6 (D) and log plasma fetuin-A concentration. Both albumin (Pearson=0.28, p = 0.03) and calcium (Pearson=0.30, p=0.022) were significantly postively associated with fetuin-A concentration. Interleukin-6 displayed a significant negative association (Pearson=-0.483, p=0.005) and interleukin-10 a negative trend (Pearson=-0.21, p=0.061) .

4.2.2.2 Tumour site is distinguishable by Fetuin-A and White Cell Count

Patients in the CRC PBL group with rectal tumours (n=22) were associated with higher circulating concentrations of fetuin-A (log median=1.52/median=33.5), whereas those with colonic tumours (n=42) were associated with lower concentrations (log median=1.45/median=28.7) (p=0.045) (Figure 4.3a). Further comparison of the clinico-pathological differences between colonic and rectal tumours showed that colonic tumours

were significantly associated with an increased white cell count (median=7.9) compared with rectal tumours (median=6.8) (p=0.011, Figure 4.3b).

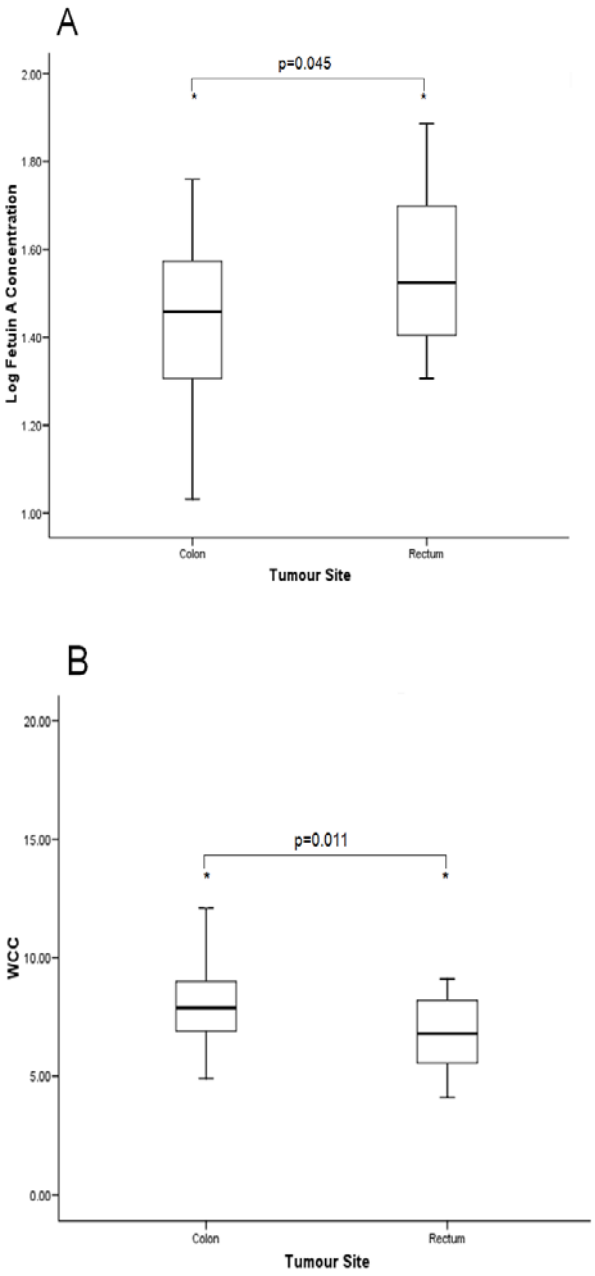

Figure 4.3: Differentiation of tumour site in the CRC PBL group using plasma fetuin-A (A) and white cell count (B). Patients with rectal cancers had significantly higher levels of fetuin-A (p=0.045) and a significantly lower white cell count (p=0.011) when compared with colon cancer patients.

4.2.3 Correlation of CRC PBL telomere length with markers of systemic inflammation

TL was again analysed both as a continuous and categorical variable. Patients were categorised into those with short telomeres (RelT/S < 0.55, shortest quartile) and those with long telomeres (RelT/S > 0.55). No significant relationship was observed between TL and CRP, pro-inflammatory cytokines (IL-6, IL-10, VEGF) or mGPS as analysed as a continuous or categorical variable (Table 4.3).

Table 4.3: Table displaying the relationship between CRC PBL telomere length and factors involved in systemic inflammation.

Systemic Inflammation

	Median (range)/ No Patients	***Correlation with Telomere Length (p<0.05)***
C-Reactive Protein	7.5 (0.40-95.0) mg/l	0.043 (0.743)‡
IL-6	6.5 (1.75-39.8) pg/ml	-0.031 (0.814)‡
Il-10	11.2 (5.68-34.82) pg/ml	-0.092 (0.490)‡
VEGF	84.75 (7.28-952.96) pg/ml	0.089 (0.503)‡
Neutrophil:Lymphocyte (NLR)	0.32 (0.04-0.94)	**0.047**¶
mGPS		
0	42 (65.6%)	NS
1	13 (20.3%)	NS
2	9 (14.1%)	NS

‡ Relationship displayed as Pearson correlation with telomere length as continuous variable (p<0.05).
¶Significant difference in NLR between short (Rel T/S <0.55, NLR 0.39 (IQR 0.90)) and long (Rel T/S >0.55, NLR 0.28 (IQR 0.71)) telomere group (Mann-Whitney 0.047).

However, there was a significant relationship between patients with short TL when analysed as a categorical variable and an elevated neutrophil:lymphocyte (NLR=0.39 (IQR 0.90) Vs NLR=0.28 (IQR 0.71)) (Mann-Whitney, p=0.047, Figure 4.4, Table 4.3). Thus, indicating higher levels of systemic inflammation in CRC patients with shorter TL, hence a potential association between inflammation and accelerated biological ageing.

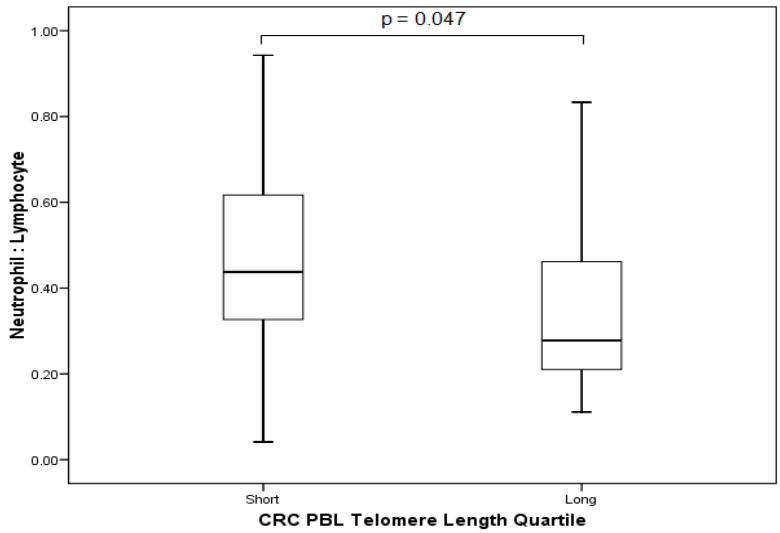

Figure 4.4: Box plot displaying the significant difference in NLR between the short (relative T/S <0.55) and long telomere (relative T/S > 0.55) group, Mann-Whitney p=0.047.

4.3 Discussion

As indicated in the previous chapter there is clear evidence of accelerated biological ageing in the form of telomere attrition in the CRC patients under investigation. As outlined in the introduction to this thesis *(Introduction section 1.3.3)* there are a number of factors which have been shown to play a role in the determination of TL. An attempt was therefore made to correlate TL with circulating factors involved in redox control and systemic markers of inflammation. Any potential association could indicate a mechanistic link in the determination of TL and hence a role in the pathogenesis of CRC.

4.3.1 Correlation of telomere length with factors involved in redox control

4.3.1.1 Antioxidant Vitamin Status

Various investigators, using a number of experimental modalities, have identified damage induced by reactive oxygen species and oxidative stress as a key determinant of telomere erosion rates (von Zglinicki 2002, Serra *et al,* 2003). The relationship between redox state and telomere dynamics is likely to be a complex one involving interaction between a wide array of genetic and environmental factors. The potential role of disordered redox state in this study was determined by measuring plasma levels of anti-oxidant vitamins,

micronutrient trace element status and factors involved in calcium homeostasis including, fetuin-A (a circulating calcium binding protein). Despite multi-modal analysis no significant relationship was demonstrated between TL and anti-oxidant status in this group of CRC patients. However, studies ranging from cell culture analysis to population based studies, have shown that anti-oxidants can protect against free radical induced telomere attrition. Alpha-tocopherol (vitamin E) can protect against H_2O_2 induced telomere damage in cultured fibroblasts, an effect dependent on telomerase activity (Makpol *et al,* 2010). Patients with type 2 diabetes mellitus, a condition associated with oxidative stress, display evidence of telomere attrition. This effect is mediated by plasma antioxidant status and genotypic variation in the expression of UCP-2, a gene involved in mitochondrial production of ROS (Salpea *et al,* 2010). In a group of healthy female study participants longer TL was associated with increased intake of multivitamins which included vitamin C and E (Xu *et al,* 2009). Specific to cancer patients, Shen *et al* (2009) report that breast cancer risk was significantly associated with shorter TL and that this risk was increased by reduced dietary intake of antioxidant vitamins (Shen *et al,* 2009). These studies indicate that the increased cancer risk associated with telomere attrition could in part be mediated by altered antioxidant status. However, it is unlikely that this current study is sufficiently powered to uncover any association between TL and plasma antioxidant status in this group of CRC patients. One advantage it does have is that determination of plasma antioxidant status is a potentially more objective assessment than dietary questionnaire. Hence studies using this methodology such as the one described above are potentially unreliable.

4.3.1.2 Trace Element Status

Similar to plasma antioxidant status, analysis of TL with regards to levels of trace element divalent cations namely; Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+} and Se^{2+} did not reveal any significant association. The potential for transition metals such as copper and iron to regulate TL by disrupting redox potential is intuitive when the Haber-Weiss and Fenton reactions are considered. In the presence of reducing agents these metal ions catalyse the production of superoxide anions (O_2^-), H_2O_2 and hydroxyl radicals *in vivo*, all potentially damaging reactive oxygen species. Copper ions have previously been shown to induce telomere attrition, an effect abrogated by subsequent cell culture with a copper ion binding molecule (Bar-Or *et al,* 2001). The effect of cancer risk predicted by the serum level of these metal ions has been investigated in the past, however never in the context of telomere attrition.

Although iron has not been shown to alter TL *in vitro* (Liu *et al,* 2004), there is some evidence that increased iron levels can increase cancer risk by altering redox state (Kabat & Rohan 2007). Magnesium and selenium ions on the other hand are considered to have anti-oxidant capabilities and hence a protective function over TL. Rats fed a magnesium deficient diet display evidence of accelerated telomere attrition rates in liver tissue samples and cultured cells, with lower activity levels of measured antioxidant enzymes. The authors conclude that magnesium deficiency leads to oxidative stress, apoptosis and accelerated ageing. Unfortunately there was no mention of cancer incidence in these animals (Martin *et al,* 2008). Selenium has also been shown to exert a protective effect over TL at the *in vitro* level, an effect mediated through increased telomerase activity (Liu *et al,* 2003). The effect of zinc on TL is unclear. *In vitro* treatment of hepatoma cells with zinc caused telomere attrition (Liu *et al,* 2004), however there are no studies correlating *in vivo* TL with zinc levels in cancer patients. Although the assessment of trace element status in this study is made from plasma samples it may be that a more accurate form of intra-cellular determination may give a more physiological depiction.

4.3.1.3 Calcium Homeostasis

Analysis has not revealed any significant relationship between PBL TL and plasma calcium concentration. Disordered calcium homeostasis could impact on TL through an increase in free radical generation and subsequent oxidative damage. Excessive calcium has been shown to increase mitochondrial free radical generation through a number of mechanisms including; enhanced citric acid cycle activity, increased NADH formation, activation of ROS generation enzymes such as α-ketoglutarate dehydrogenase and promoting the loss of cytochrome *c* (Kowaltowski *et al,* 2009). These sequelae of increased calcium concentration at the mitochondrial level could reflect a relative inability to bind free calcium caused by lower fetuin-A, with subsequent promotion of a pro-oxidative state and leading to accelerated telomere attrition. Excess calcium has been shown to negatively impact on prostate cancer outcome. Potential reasons for this include the fact that both calcium and parathyroid hormone (release of which is stimulated by excess physiological calcium) can promote proliferation and metastasis of prostate cells *in vitro,* however an impact on redox state cannot be discounted (Skinner & Schwartz 2009).

Some recent studies have uncovered a potential relationship between levels of vitamin D and the ageing process, specifically with TL. Analysis of serum vitamin D levels in the

context of TL in the CRC patients in this study did not reveal any significant association. This was also the case when vitamin D levels were correlated with patient clinic-pathological parameters. Correlation of TL with vitamin D levels in a large population-based cohort of twins has revealed significant strong positive relationship, which became stronger as vitamin D level increased. Furthermore, Vitamin D levels were negatively correlated with CRP in the study population. It therefore appears that the protective effect of Vitamin D over TL results from its ability to act as an anti-inflammatory mediator. Indeed vitamin D receptors are ubiquitously expressed on both T and B cells, and can down-regulate cytokine production producing an anti-inflammatory effect (Richards *et al*, 2007). Thus the effect of Vitamin D on TL appears to be independent of its role in the maintenance of plasma calcium levels. However, these studies need to be interpreted with caution as evidence has also pointed to promotion of a premature ageing phenotype in mutant mice (klotho$^{-/-}$ and FGF23$^{-/-}$) exposed to hypervitaminosis D_3 through dietary modification and genetic manipulation of the vitamin D receptor. Although, TL was not specifically examined in these models expression of ageing-related genes was altered (Tuohimaa 2009). The current study was not sufficiently powered to fully delineate the relationship between vitamin D, telomere attrition and CRC, however given recent intriguing experimental evidence a potentially manipulatable determinant of TL in cancer patients may exist.

Within the CRC PBL group patients with lower fetuin-A levels had shorter telomeres. These findings are consistent with those in CKD patients where there is an established link between low fetuin-A levels, short TL and reduced anti-oxidative capacity. Indeed, in this group of patients TL was associated with increased mortality, independent of age and sex but dependent on fetuin-A levels (Carrero *et al*, 2008). The relationship between TL and fetuin-A indicates biological ageing is associated with reduced redox capacity within the blood of CRC patients. Interleukin 6 (IL-6), a pro-inflammatory cytokine, alters the gene expression and synthesis of fetuin-A by hepatocytes, similar to its action on albumin (Gangneux *et al*, 2003). This study showed that decreasing fetuin-A levels correlates with decreasing albumin concentration and increasing IL-6 and IL-10 levels, supporting the hypothesis of inflammation-dependent down-regulation of fetuin-A expression. These observations concur with those described in patients with renal failure on dialysis (Hermans *et al*, 2007) and also with results from a rodent model of lethal systemic inflammation where fetuin-A exerted a protective role (Li *et al*, 2011a). However, the

relationship between fetuin-A is not a straightforward one given the finding of elevated levels in patients with previous myocardial infarction and obesity (Voros *et al*, 2011).

In patients with glioblastoma, the most commonly occurring brain tumour low serum fetuin-A levels predicted poor survival (Petrik *et al*, 2008). Moreover, Rho *et al* (2009) report differential expression of fetuin-A between lung adenocarcinoma and adjacent normal tissue with both total protein and mRNA abundance reduced in cancer samples (Rho *et al*, 2009). These findings, in conjunction with our own observations, lead to the intriguing possibility of utilising fetuin-A as a prognostic/predictive marker for tumours from a histologically varied origin. Work is required to determine whether it is by virtue of its role in calcium homeostasis and hence redox state, that fetuin-A contributes to the determination of TL. Clearly comparison of fetuin-A levels between cancer patients and healthy controls would also be essential in any further investigation of the role of fetuin-A in cancer.

Rampazzo et al (2010) have identified right-sided colonic tumours as having shorter TL than left-sided and rectal cancers, which may result from an alteration in mismatch repair pathways (Rampazzo *et al*, 2010). The molecular and clinical characteristics of right and left colon cancers are well established with right sided tumours classically associated with microsateillite instability and alteration in CpG island methylator phenotype, and left sided cancers with chromosomal instability (Iacopetta 2002). The observation that plasma fetuin-A levels vary with the anatomical site of the primary tumour is pertinent and may reflect differences in the systemic oxidative-inflammatory milieu as a result of differing molecular characteristics in the anatomical position of the tumour. This could particularly reflect differing rates of telomere attrition which were not elicited in the current study as no relationship between tumour site and TL was determined. The pre-operative management of colonic and rectal cancers in the context of chemo-radiotherapy differs, hence, further molecular differentiation of colon and rectal cancers could lead to the discovery of new therapeutic targets thereby improving the outcome of rectal cancer. Obviously, further work in a larger patient group is required to substantiate these preliminary findings and determine ’cause and effect’ relationship between anatomical differences in tumour site and telomere dynamics.

4.3.2 Correlation of Telomere Length with Markers of Inflammation

Inflammation is an important determinant of TL which has been implicated in the ageing process and as a universally detrimental factor at all stages of the neoplastic process. We therefore sought to correlate TL with common bio-haematological markers of inflammation, in addition to pro-inflammatory cytokines.

Cellular senescence is a permanent state of growth arrest and hence a potent mechanism of tumour suppression. The triggers of senescence *in* vivo include critical telomere attrition, activation of oncogenes, oxidative stress, genotoxic stress and some therapeutic interventions for example irradiation and chemotherapy. Recent evidence indicates that senescent cells secrete a multitude of signalling factors, termed the "senescence associated secretory phenotype" (SASP) (Coppe *et al,* 2008). These signals are mostly pro-inflammatory and include factors such as IL-1α and β, IL-6 and IL-8. The SASP provides an intuitive explanation of our observed association between short TL and systemic inflammation, indicated by a raised neutrophil:lymphocyte ratio. Interestingly, patients with ulcerative colitis an inflammatory condition of the colon who exhibit an increased risk of CRC display evidence of telomere attrition in leucocytes (Risques *et al,* 2008a). Even minute quantities of pro-inflammatory cytokines released by populations of senescent cells in biologically aged individuals could stimulate a more chronic systemic inflammation by virtue of positive feedback loops. At the peri-tumoural level these factors could also act in a paracrine fashion to create an environment where tumour cells can flourish by stimulating hyperproliferation, de-differentiation, immune evasion, migration and invasion (Davalos *et al,* 2010).

It is therefore likely that a complex inter-relationship exists between telomere dynamics and inflammation via positive feedback loops. As pro-inflammatory signalling pathways are up-regulated and haemopoetic stem cells stimulated to differentiate into lymphocytic cells repeated rounds of division will contribute to the acceleration in telomere attrition demonstrated in PBLs. Increased numbers of senescent cells would therefore lead to an increase in the pro-inflammatory milieu and perpetuation of the pro-inflammatory state contributing to the acceleration of biological ageing.

CHAPTER 5: SIRTUIN EXPRESSION IN COLORECTAL CANCER

5.1 Introduction

Colorectal cancer is the third most common cancer in the UK and is responsible for 16,000 deaths every year (Cancer Research UK 2010). Improving understanding of the key mechanisms underpinning the disease process could lead to the discovery of novel prognostic biomarkers or targets for therapeutic intervention. The link between cancer and ageing seems intuitive given that age is a risk factor common to most solid organ cancers, especially those of an epithelial origin (DePinho 2000). Current lines of investigation linking the two processes include the contribution of telomere biology (Shay & Wright 2010, Maxwell *et al*, 2011) and manipulation of nutrient sensing pathways, such as mammalian target of rapamycin (mTOR) (Zoncu *et al*, 2011). The sirtuin family of genes have been intensely investigated due to their diverse cellular functions and ability to influence longevity in certain organisms.

Variability in the sub-cellular localisation of the sirtuins is reflected in the variety of critical cellular functions to which the sirtuins contribute. At the mitochondrial level, SIRT3 and SIRT4 have been shown to regulate the activity of acetyl-CoA (Hallows *et al*, 2006) and glutamate dehydrogenase (Haigis *et al*, 2006) respectively, whereas SIRT5 is implicated in the control of the urea cycle (Nakagawa & Guarente 2009). SIRT6 plays an important role in the maintenance of genomic integrity and DNA damage repair (Mostoslavsky *et al*, 2006). SIRT6 has also been shown to modulate telomeric chromatin and hence function by histone H3 deacetylation (Michishita *et al*, 2008a). SIRT1 contributes to glucose homeostasis by stimulating hepatic gluconeogenesis (Rodgers *et al*, 2005) and insulin release from pancreatic β-bells (Moynihan *et al*, 2005). In addition, by virtue of its ability to deacetylate a number of non-histone proteins such as p53, FOXO, NF-κB and Ku70, SIRT1 controls the response to cellular stress and dictates cellular fate by modulating apoptotic and DNA repair pathways (Yang *et al*, 2005, Jeong *et al*, 2007, Ghosh *et al*, 2007, Yi& Luo 2010). SIRT7 appears to regulate cell growth in response to changing metabolic conditions by stimulating ribosome biogenesis (Ford *et al*, 2006). SIRT2 has been shown to exert control over the cell cycle by deacetylating α-tubulin, whilst its levels increase during mitosis with overexpression delaying mitosis (North & Verdin 2007b).

These key cellular processes over which the sirtuins exert a degree of control all play a role in normal organismal ageing. It therefore stands to reason that dysregulation of sirtuin expression could alter lifespan and contribute to the development of age related diseases. Indeed SIRT6 knockout mice die young displaying signs of progeria, whilst SIRT1 has been implicated in the lifespan extension of model organisms conferred by caloric restriction (Cohen *et al,* 2004b, Mostoslavsky *et al,* 2006). The sirtuin family have been implicated in the pathophysiology of age-related conditions including cancer. Attempting to delineate the relationship between the sirtuin family and cancer is proving complex. This is exemplified by the fact that despite the plethora of studies in numerous experimental systems there is still some dubiety as to whether they function primarily as tumour promoters or suppressors. Reflecting this dichotomous position SIRT1 has been shown to be upregulated in leukaemia, prostate and skin cancer (Bradbury *et al,* 2005, Hida *et al,* 2007, Huffman *et al,* 2007), while downregulated in colorectal cancer (Firestein *et al,* 2008). SIRT2 and SIRT7 have been implicated in the development of gliomas (Hiratsuka *et al,* 2003) and human papillary thyroid cancer cell lines respectively (de Nigris *et al,* 2002).

We have previously hypothesised that the sirtuins link Mitochondria and the Telomere nucleoprotein complex with Ribosome biogenesis (Shiels & Davies 2004). This provides a plausible mechanistic pathway where the energy balance of a cell in stress is sensed and the requisite damage response mechanisms initiated. Aberrant sirtuin expression could, therefore, upset the balance of these factors hence tipping the cell into crisis if key repair pathways could not be initiated. Moreover, the inability of the cell to respond to DNA damage in particular could lead to deleterious chromosomal events and potentially carcinogenesis. It is therefore conceivable that the sirtuins play a key role in the development and progression of the cancer process.

Chapter Aims

- To determine the relative transcription levels of SIRT1-7 in colorectal cancer tissue and adjacent normal mucosal tissue, identification of a positive or negative relationship between the two tissue types could provide further evidence to support a tumour suppressive or promoting role for the sirtuins in CRC.

- Expression data was then correlated with a variety of commonly recorded clinico-pathological, biochemical and haematological variables. Therefore, we aimed to place the biology of ageing and cancer in a clinical context.

- Characterise the relationship between sirtuin expression and TL in CRC tissue.

- Correlation with pathological and cancer outcome was also made to allow evaluation of sirtuin expression as a marker of prognosis.

5.2 Results

5.2.1 Differential Expression between Colorectal Tumour and Adjacent Normal Tissue

The overall aim of this study was to create an expression profile for SIRT 1-7 from colorectal cancer specimens and paired adjacent normal tissue. There was a universal significant attenuation in relative expression levels of all seven sirtuins in the tumour tissue when compared with normal tissue (SIRT3 n=43, SIRT5 n=46, SIRT1 and SIRT6 n=47, SIRT2 and SIRT7 n=48, SIRT4 n=49; Figure 5.1). All but SIRT 2 ($p=0.026$) displayed a significant difference in expression at the $p<0.001$ level. Within the tumour samples the lowest expression levels were evident with SIRT4 (0.14, IQR 0.18) and highest with SIRT5 (0.55, IQR 0.51). As indicated in Figure 5.1 SIRT1 (0.41, IQR 0.22), SIRT2 (0.42, IQR 0.27) and SIRT3 (0.39, IQR 0.21) all displayed similar expression levels within the tumour tissue (Kruskal-Wallis, $p=0.962$), while there was a significant difference in the expression of the mitochondrial sirtuins, with SIRT4 displaying the lowest relative transcription levels (Kruskal-Wallis, $p<0.001$). Analysis of cancer tissue subgroups revealed differences in the relative expression between rectal tumour tissue samples from patients who had undergone neo-adjuvant therapy prior to their index procedure (*Effect of Neo-Adjuvant Therapy on SIRT Relative Expression 5.2.3.1*). To prevent these differences confounding the overall results, therefore giving a more accurate representation of relative expression these samples were excluded from the SIRT transcriptional analysis presented above.

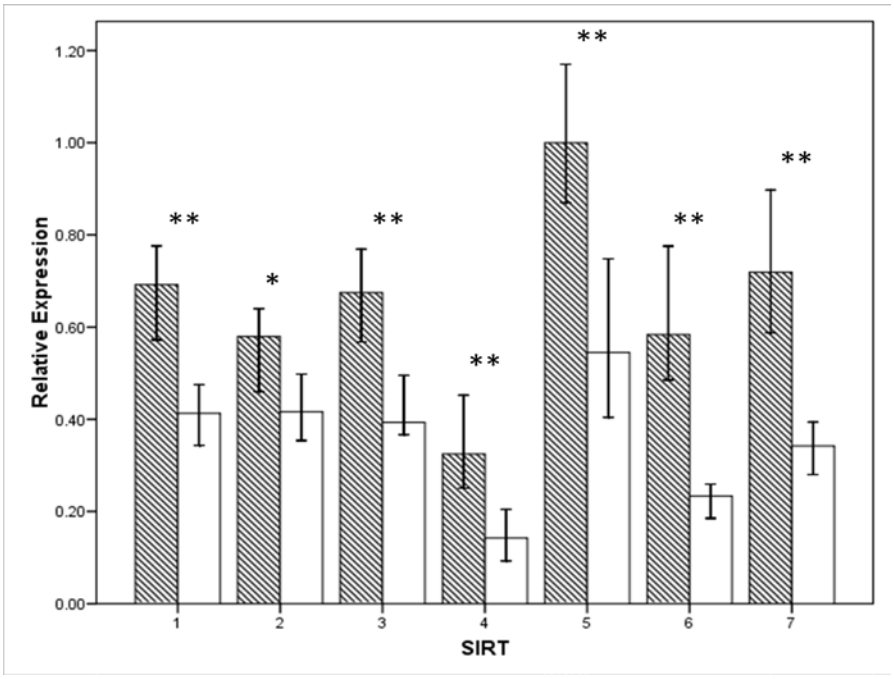

Figure 5.1: Bar chart displaying the median expression level (± 95% C.I) of each of the sirtuins in colorectal cancer tissue (solid bar) and adjacent normal tissue. Expression level was significantly reduced in all seven sirtuins in cancer tissue compared with normal. (Wilcoxon signed rank Test, **P<0.0001, *P=0.003).

5.2.2 Inter-relationship between sirtuins in tumour and normal tissue

To determine if there was any inter-relationship between individual sirtuin relative expression levels from tumour and normal tissue, cross-correlation analysis was performed. The various correlations are outlined in Table 5.1, with a number of strong positive correlations revealed. Of particular note, within the tumour samples SIRT1 was strongly positively correlated with SIRT2 (Pearson r = 0.827), SIRT3 (Pearson r = 0.647) and SIRT4 (pearson r = 0.850), all $p<0.001$. In addition SIRT3 correlated with all but SIRT7 at the $p < 0.001$ level. There were some major differences in the correlation analysis from the normal tissue samples, with the loss of all significant associations between SIRT5, SIRT6 and the remainder of the sirtuins (Table 5.1). There was, however, an association gained between SIRT3 and SIRT7 (Pearson r = 0.665, $p < 0.001$).

Table 5.1: Table displaying correlations between SIRT1-7 in the CRC tumour (A) and normal (B) samples.

A Tumour

	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1 Pearson r *p*-value	0.827 **<0.001**	0.647 **<0.001**	0.850 **<0.001**	0.319 0.033	0.447 **0.002**	0.396 0.006
SIRT2 Pearson r *p*-value		0.610 **<0.001**	0.753 **<0.001**	0.475 **0.001**	0.414 0.004	0.453 **0.001**
SIRT3 Pearson r *p*-value			0.609 **<0.001**	0.568 **<0.001**	0.531 **<0.001**	0.438 **0.002**
SIRT4 Pearson r *p*-value				0.216 0.170	0.420 0.006	0.209 0.178
SIRT5 Pearson r *p*-value					0.463 **0.001**	0.628 **<0.001**
SIRT6 Pearson r *p*-value						0.635 **<0.001**

B Normal

	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1 Pearson r *p*-value	0.902 **<0.001**	0.509 **<0.001**	0.758 **<0.001**	0.222 0.143	0.056 0.711	0.140 0.348
SIRT2 Pearson r *p*-value		0.665 **<0.001**	0.834 **<0.001**	0.151 0.318	0.003 0.983	0.165 0.262
SIRT3 Pearson r *p*-value			0.744 **<0.001**	0.386 0.008	0.363 0.012	0.665 **<0.001**
SIRT4 Pearson r *p*-value				0.199 0.207	-0.042 0.793	0.244 0.116
SIRT5 Pearson r *p*-value					0.257 0.089	0.364 0.013
SIRT6 Pearson r *p*-value						0.888 **<0.001**

$P \leq 0.002$ deemed significant after Bonferroni correction (emboldened *p* value = significant)

5.2.3 Differentiation of Tumour Site

An attempt was made to differentiate between tumour site according to sirtuin relative expression level. Tumour site was defined according to operative findings and pathological examination of the resected specimen. Rectal cancers were deemed to lie below the peritoneal reflection with colon cancers above. Colon cancers were further divided into proximal and distal according to their position in relation to the splenic flexure. Analysis revealed that colon cancer specimens had significantly lower levels of SIRT2 expression (Figure 5.2) (Mann-Whitney, p=0.021) with SIRT4 levels also reduced, the relationship displaying a trend towards significance (Mann-Whitney, p=0.056). Further differentiation into proximal and distal colon cancers revealed a sequential reduction in SIRT2 expression levels from the rectum to the proximal colon, which displayed a clear trend but did not reach significance (Kruskal-Wallis, p=0.072). Interestingly, SIRT2 expression in the normal tissue samples was also significantly different between colonic and rectal tissue samples in a similar pattern to that seen in tumour tissue (Mann-Whitney, p=0.025).

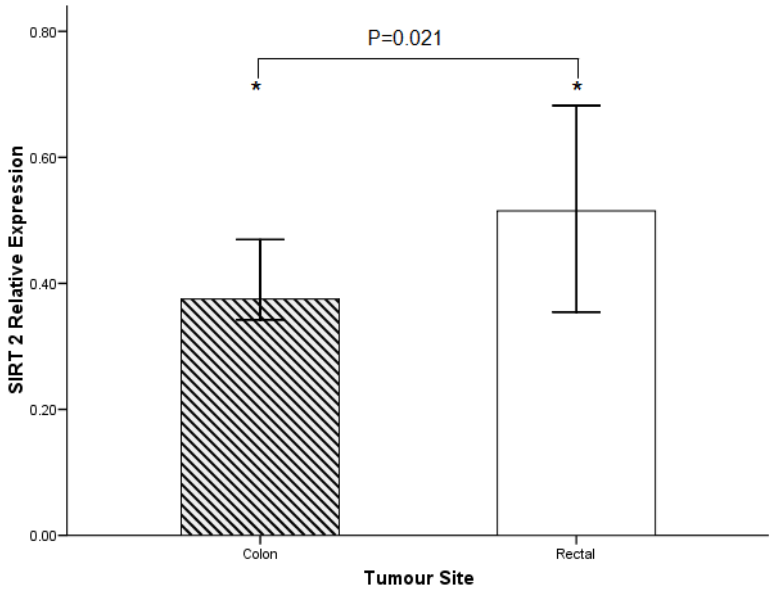

Figure 5.2: Differentiation of tumour site using median SIRT2 (± 95% C.I.) relative expression. Patients with colon cancer had significantly lower levels of SIRT2 compared to those with rectal cancer (Mann-Whitney, p=0.021).

5.2.3.1 Effect of Neo-adjuvant therapy on SIRT relative expression

Using the small group of rectal cancer patients (n=6) who had undergone neo-adjuvant therapy an attempt was made to determine if pre-operative chemo/radiotherapy had an effect on the expression on SIRT1-7 in the cancer and normal samples. As displayed in Table 5.2 there was a significant increase in the relative expression of SIRT1, SIRT3 and SIRT4 in the cancer tissue samples when comparing non- and neo-adjuvantly treated patients. This was most pronounced for SIRT3 where there was a marked increase in expression in tumours exposed to neo-adjuvant therapy (0.85 (±0.36) Vs 0.41 (±0.21), $p<0.001$). Within the normal samples neo-adjuvant therapy significantly increased SIRT6 expression (0.97 (±0.49) Vs 0.54 (±0.51), $p = 0.033$). Moreover, neo-adjuvant therapy appears to reverse the differential expression of SIRT1-SIRT4, between tumour and normal tissue with higher relative transcriptional levels in the tumour samples (Table 5.2). However, none of the differences reached a significant level. For a number of reasons these data should be interpreted with a degree of caution. The small sample size is an obvious limitation as is the lack of detail regarding the specificities of the therapeutic regimens employed. Importantly, there are also no direct control pre-neo-adjuvant treatment samples from which to make comparisons therefore it is possible SIRT levels could have been elevated in the relevant samples prior to neo-adjuvant therapy.

Table 5.2: Table displaying the difference in relative expression of SIRT1-7 in each of the cancer and normal tissue source, further stratified according to neo-adjuvant therapy.

	Tumour			**Normal**		
	Neo-adjuvant	***Non neo-adjuvant***	***P (<0.05)***	***Neo-adjuvant***	***Non neo-adjuvant***	***P (<0.05)***
SIRT1	0.69 (0.36)*	0.43 (0.22)	**0.022**	0.53 (0.68)*	0.69 (0.42)	0.845
SIRT2	0.64 (0.56)*	0.42 (0.27	0.106	0.50 (0.75)*	0.54 (0.41)	0.501
SIRT3	0.85 (0.40)*	0.41 (0.21)	**<0.001**	0.80 (0.67)*	0.65 (0.43)	0.088
SIRT4	0.38 (0.30)*	0.14 (0.17)	**0.026**	0.32 (0.96)*	0.32 (0.31)	0.960
SIRT5	0.68 (0.80)	0.56 (0.19)	0.408	1.10 (1.08)	0.98 (0.63)	0.854
SIRT6	0.28 (0.10)	0.23 (0.12)	0.263	0.92 (0.49)	0.54 (0.51)	**0.033**
SIRT7	0.52 (0.39)	0.35 (0.21)	0.106	0.96 (0.66)	0.67 (0.55)	0.133

All values displayed as median (IQR). N=6 in the neoadjuvant treated group
Differences between the two groups analysed by Mann-Whitney, $p<0.05$ deemed significant.
*The relationship between relative expression has been reversed with higher levels within the tumour samples compared with the normal (SIRT1-4). None of these reached significance (Wilcoxon signed rank test p>0.05)

5.2.4 Association with Clinico-pathological parameters

We attempted to delineate any relationship between sirtuin expression and commonly recorded patient clinico-pathological characteristics. In order to achieve this, our analysis was performed with SIRT1-7 expression level included as both a continuous or categorical variable where appropriate. Categorisation into high or low expression groups was achieved by dividing expression level around the median value for the respective sirtuin. Analysis of expression levels of SIRT4 in tumour samples revealed a significant difference in expression between patients with lymph node positive and negative disease (Mann-Whitney, p=0.027), with higher SIRT4 levels apparent in patients with node positive disease (Figure 5.3). There were no other significant relationships between SIRT1-7 and any of the other parameters outlined in Table 2.2.

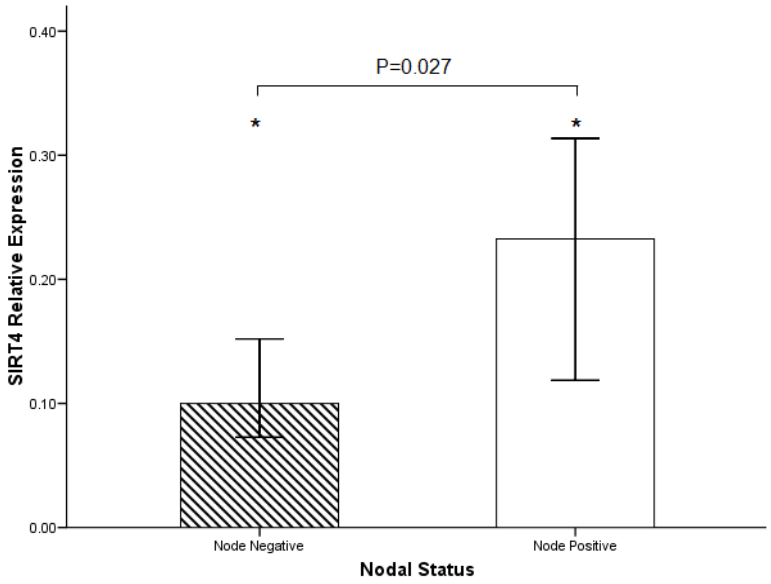

Figure 5.3: Bar chart demonstrating the significant difference in median SIRT4 expression (± 95% C.I) in cancer tissue differentiated by pathological nodal status (Mann-Whitney, p=0.027).

5.2.5 Correlation with Survival

The median survival of the patient group was 54.1 months (0.33-84.40). Survival analysis was performed using the sirtuin expression data in the categorical form as before. Analysis revealed that higher expression levels of SIRT4 were associated with a poorer outcome in terms of survival, with more deaths in this group compared with low expression levels. (log rank p =0.025, Figure 5.4). There was no association with survival for the remaining sirtuins.

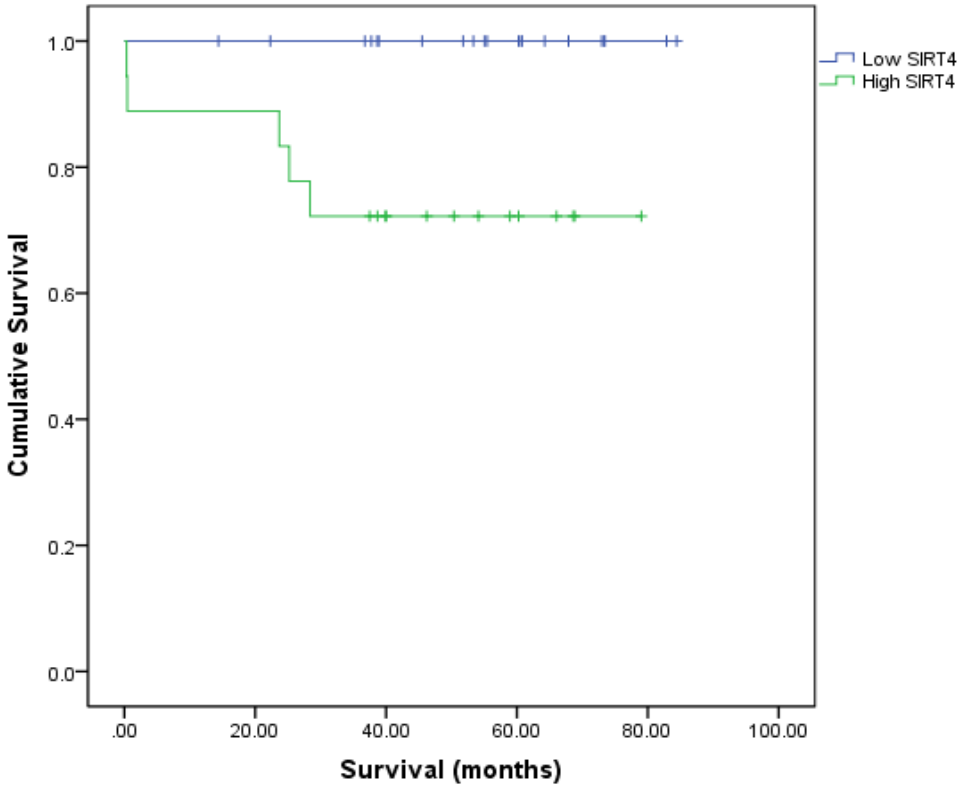

Figure 5.4 Kaplan-Meier survival curve comparing survival with SIRT4 expression at high or low levels. Significantly more deaths in the SIRT4 expression group (log rank p=0.025).

5.2.6 Sirtuin Expression and Indices of Biological Ageing

5.2.5.1 Correlation with Systemic Inflammation

On analysis of sirtuin expression levels in the context of common indices of inflammation (WCC, neutrophils, CRP, albumin and mGPS) there was a significant relationship between CRP level and relative expression levels of SIRT2, SIRT3, SIRT4 and SIRT7 in the normal colorectal tissue. Whereby, patients with higher levels of CRP and hence higher levels of inflammation displayed lower expression levels of the respective sirtuins (Mann-Whitney, SIRT2 p=0.025, SIRT3 p=0.012, SIRT4 p=0.021 and SIRT7 p=0.047) (Figure 5.5A). Consistent with these findings there was an inverse relationship between CRP and both SIRT2 (Pearson r -.346, p=0.093) and SIRT4 (Pearson r -.323, p=0.088) (Figure 5.5B), which did not reach significance. There was no relationship between tumour sirtuin expression level and indices of inflammation.

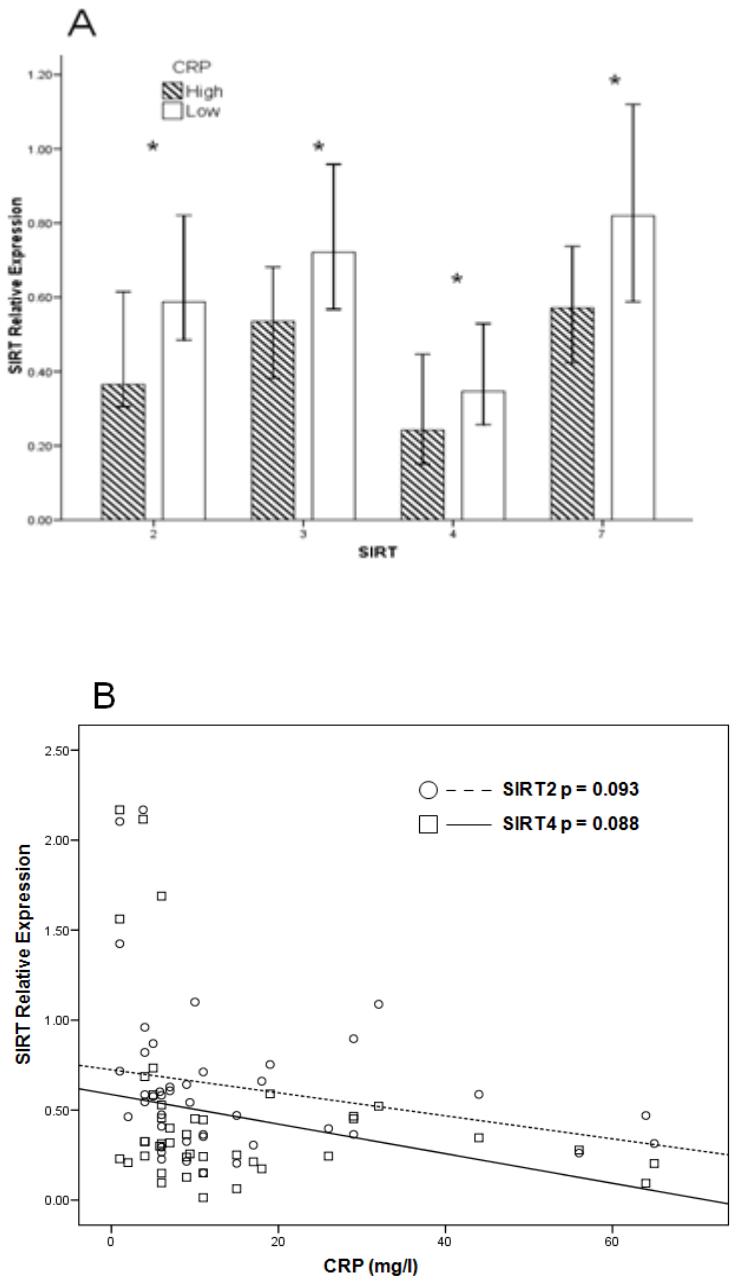

Figure 5.5: (A) Bar chart indicating the significant difference in expression levels in normal colorectal tissue of SIRT2 (p=0.025), SIRT3 (p=0.012), SIRT4 (p=0.021) and SIRT7 (p=0.047) between those patients with high (>10 mg/l) and low (<10 mg/l) levels of C-reactive protein ± 95% C.I. (B) Scatter plot demonstrating the inverse relationship between SIRT2 (Pearson r -.251, p=0.093), SIRT4 (Pearson r -.266, p=0.088) and C-reactive protein.

5.2.5.2 Correlation with chronological and biological ageing

No significant relationship was demonstrated between chronological age and any of the individual sirtuins, in either the tumour or normal tissue samples. DNA was available for determination of TL in twenty-nine matched samples from the original batch of thirty-two. As was previously reported TL was significantly shorter in tumour samples (median=0.41,

IQR=0.26) when compared with normal adjacent normal tissue (median=0.63, IQR=0.22) (Mann-Whitney, p<0.001). Thus, indicating tumour cells were more biologically aged than adjacent normal cells. Expression levels of all seven sirtuins were correlated with TL from both the cancer and normal tissue cohort. Analysis revealed a significant inverse relationship between TL and SIRT3 relative expression in cancer tissue (Pearson r -.353, p=0.047) (Figure 5.6).

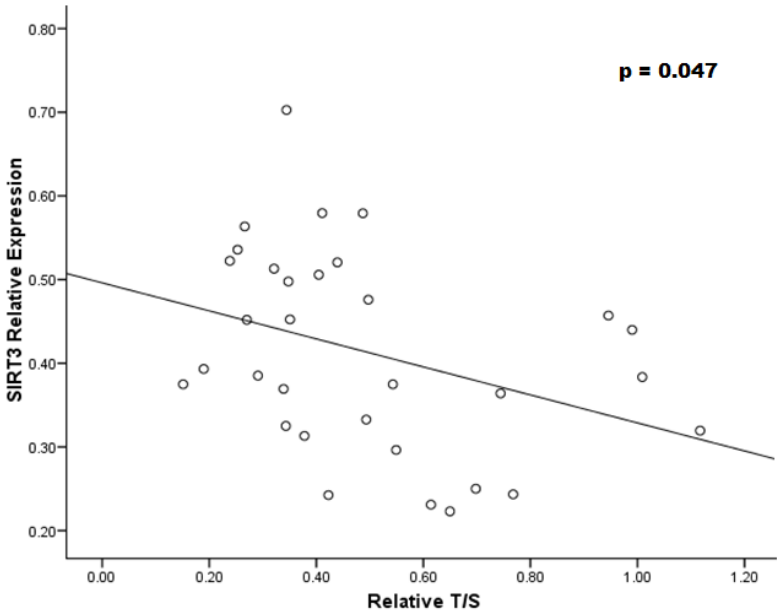

Figure 5.6: Scatter plot demonstrating the significant inverse relationship between SIRT3 relative expression and telomere length in colorectal cancer specimens (Pearson r -.353. p=0.047).

5.3 Discussion

5.3.1 Differential Relative Expression of SIRT1-7 in Tumour and Normal Tissue

We have constructed an expression profile for all seven mammalian sirtuins in the index cohort of CRC patients. These results demonstrate that relative expression levels of all seven sirtuins were significantly attenuated in tumour tissue when compared with normal

adjacent tissue. These data corroborate previous studies which have implicated the sirtuins, in particular SIRT1 as potential tumour suppressors (Kabra *et al*, 2009).

5.3.1.2 Reduced relative expression of SIRT1 in Colorectal cancer tissue

Given the pro-apoptotic effect of SIRT1 a tumour suppressive function is not difficult to rationalise. The reduced expression in the colorectal tumour samples compared with normal tissue in this study indicate a reduced apoptotic capacity and therefore reduced ability to eliminate damaged cells. Aberrancy of the SIRT1-NFκB axis could explain the tumour suppressive effect of SIRT1. SIRT1 represses NFκB and facilitates TNF-α induced apoptosis, therefore attenuation of SIRT1 expression would result in the loss of this important pro-apoptotic pathway (Yeung *et al*, 2004). Loss of SIRT1 could also promote neoplastic transformation by a reduced capacity to maintain genomic integrity. Wang et al (2008) report that not only is SIRT1 expression reduced in a variety of human cancers compared with controls but that SIRT1 deficient mice display chromosomal aberrations with a reduced ability to repair DNA double strand breaks (DSBs) characterised by diminished γH2AX, BRCA1, RAD51 and NBS1 foci (Wang *et al*, 2008a).

Consistent with our demonstration of a reduced relative expression of SIRT1 in CRC samples, two published studies have investigated the potential for SIRT1 to act as a tumour suppressor specifically in CRC. Using cultured colorectal cancer cells (HCT116 cells) Kabra *et al* (2009) report that over-expression of SIRT1 induced efficient G_1 cell cycle arrest. Treatment of these cultured cells with a specific SIRT1 inhibitor stimulated proliferation, an effect not seen in SIRT1 null MEFs. A tumour xenograft assay also demonstrated tumour growth was increased upon inoculation of athymic nude mice with HCT116 cells in which the SIRT1 gene was inactivated by shRNA. Finally, colorectal cancer specimens and controls were stained for SIRT1 revealing high expression in normal tissue and benign adenomas, with 30% of tumour samples showing less intense staining than control samples. The authors conclude that the tumour suppressive role for SIRT1 in this setting results from activation of E2F1 and hyper-phosphorylation of pRb. Attenuated expression of SIRT1 in this scenario would allow cells to by bypass senescence in the face of stress leading to increased proliferation and neoplastic transformation (Kabra *et al*, 2009).

Firestein et al (2008) have also demonstrated suppression of intestinal tumourigenesis by SIRT1 using a comprehensive array of methodologies. This group generated transgenic mice ($APC^{min/+}SIRT1^{\Delta STOP}$) which over-expressed SIRT1 in the setting of a β-catenin driven model of intestinal tumourigenesis. These animals displayed fewer tumours than controls and similar levels to $APC^{min/+}$ mice fed a calorie restricted diet. Ectopic induction of SIRT1 in a β-catenin driven model of colorectal cancer greatly reduced proliferation, giving some insight into a potential mechanism of tumour suppression. The authors also report a significant inverse correlation between nuclear SIRT1 and β-catenin in colon cancer specimens (Firestein *et al,* 2008). Analysis of the drivers of colorectal neoplasia used was not performed in our study group, however constitutive activation of the canonical Wnt/β-catenin pathway has been demonstrated in the majority of colorectal cancers (McDonald & Silver 2011). This means that the attenuation of SIRT1 in cancer cells in our group of colorectal cancer patients could in theory facilitate neoplastic transformation via de-repression of the Wnt/β-catenin pathway.

Although there is compelling evidence to support SIRT1 as a tumour suppressor one might expect that aberrant expression of genes which promote longevity should, as a by-product of encouraging cell survival lead to an increase in neoplasia in populations of dividing cells i.e. act as a oncogene. In this regard, SIRT1 can deacetylate and therefore inhibit p53 leading to the bypassing of apoptosis, a fundamental tumour suppressor mechanism (Luo *et al,* 2001, Vaziri *et al,* 2001). Consistent with this, levels of SIRT1 have been shown to be significantly elevated in human skin cancer (Hida *et al,* 2007), colon cancer (Stunkel *et al,* 2007) and prostate cancer (Huffman *et al,* 2007). Furthermore, SIRT1 deficient mice exhibit p53 hyperacetylation and increased radiation induced apoptosis (Cheng *et al,* 2003). Whilst, Zhao et al (2008) report that deleted in breast cancer-1 (DBC-1) inhibits SIRT1 activity with concomitant increased p53 functionality which is reversed by knock-down of DBC-1 using RNA-interference (Zhao *et al,* 2008). Both of these studies suggest SIRT1 expression may increase cancer risk by inhibiting p53. Dysregulation of the HIC1-SIRT1-p53 regulatory loop has also been implicated in cancer development and outcome with the finding that deregulation of this feedback loop equates with poor prognosis from lung cancer (Tseng *et al,* 2009).

5.3.1.2 Reduced relative expression of SIRT2 in colorectal cancer tissue

The data presented here pertaining to the reduced relative expression of SIRT2 in CRC specimens confirms that previously reported in glioma samples and cell lines, at both the protein and RNA level (Hiratsuka *et al,* 2003). SIRT2 is implicated in tumour formation through its role as a mitotic checkpoint regulator via modification of α-tubulin. Attenuated SIRT2 expression could therefore confer on tumour cells the ability to escape mitotic checkpoints in the face of otherwise fatal genotoxic damage. Consistent with this ectopic expression of SIRT2 exerts an anti-proliferative effect on glioma cells (Inoue *et al,* 2007, Inoue *et al,* 2009). Attenuated SIRT2 expression could result from the telomerase driven shortening of cell cycle check point time due to hyper-proliferation. As SIRT2 does not have time to function adequately its expression is therefore down-regulated.

However, the effect of SIRT2 expression on tumour cells mirror that of SIRT1 in that recent evidence has also indicated that SIRT2 inhibition can induce apoptosis in HeLa and other cancer cells caused by the accumulation of p53. An effect caused by the p38 MAPK dependent degradation of p300 and subsequent MDM2 degradation (Li *et al,* 2011). This evidence implicates SIRT2 as a promoter of tumour cell survival. Attenuation of SIRT2 function with specific inhibitors has shown promise as novel anti-cancer therapies by promoting cell cycle arrest and apoptosis in prostate, pancreatic and lung cancer cell lines (Zhang *et al,* 2009).

Clearly there is contrasting evidence relating to the role of SIRT2 in the neoplastic process. This may relate to the different model systems used as Hela cells of squamous origin will differ in their biology in comparison with neuronal cells such as those used in the glioma cell experiments described above. It is therefore difficult to extrapolate these results into the CRC setting used in our study as the histological origin, risk factor profile and molecular drivers of these tumours differs from those of CRC.

5.3.1.3 Reduced relative expression of SIRT3 in colorectal cancer tissue

SIRT3 relative expression was reduced in tumour tissue when compared with normal colorectal tissue. This finding validates those by Kim *et al* (2010) who also confirmed that SIRT3 expression was reduced in breast, testicular, glioblastoma multiforme, prostate, head and neck squamous cell, clear cell renal and hepatocellular cancer (Kim *et al,* 2010). The ability of SIRT3 to suppress damage induced by ROS seems key to its tumour

suppressor effect as isolated MEFs from SIRT3$^{-/-}$ mice show increased stress induced superoxide levels and genomic instability, augmented superoxide dismutase expression abrogated some of these effects (Kim *et al,* 2010). This intricate relationship between SIRT3 and oxidative stress is confirmed by a decrease in tumour development induced by over-expression of SIRT3 in tumour xenograft models, an effect mediated by regulation of HIF-1α (Bell *et al,* 2011). These and our data implicate SIRT3 as a tumour suppressor, an effect mediated through an integrated cellular response encompassing key mitochondrial mechanisms to suppress ROS generation and subsequent neoplasia inducing genotoxic damage.

In contrast to the evidence supporting a tumour suppressor role for SIRT3 a further recent study has reported an over-expression of SIRT3 in oral squamous cell carcinoma tissue microarrays and cell lines. *In vivo* models showed reduced tumour burden upon transgenic down-regulation of SIRT3 (Alhazzazi *et al,* 2011). These findings indicate this particular tumour type requires SIRT3 for its ongoing survival.

5.3.1.4 Reduced relative expression of SIRT4 and SIRT5 in colorectal cancer tissue

All data pertaining to SIRT4 and SIRT5 relates to their role in the regulation of fuel utilisation, mitochondrial metabolism, control of the urea cycle and control of insulin secretion (Ahuja *et al,* 2007, Haigis & Sinclair 2010). Our demonstration of reduced expression in CRC tissue is therefore the first description of aberrant SIRT4 or SIRT5 expression in cancer. The potential relationships can only be speculated upon given the lack of pertinent data. The reduced expression may reflect a compensatory mechanism by the tumour to regulate energy utilisation in the face of variability in supply depending the degree and functionality of neoangiogenesis. The lack of identification of functional relationship between SIRT4, SIRT5 and any factors know to regulate cell survival makes it unlikely they act in a tumour suppressive fashion, however this may change as enhancement of the understanding of these relationships develops.

5.3.1.5 Reduced relative expression of SIRT6 in colorectal cancer tissue

Demonstration of a reduced expression of SIRT6 in colorectal tumour samples compared with normal tissue is confirmatory of a tumour suppressive function of SIRT6. A number of histone and non-histone interactions explain this function:

1. As described previously SIRT6 has a well recognised effect on DNA repair and hence is regarded as maintainer of genomic integrity. This effect is mainly mediated through the ability of SIRT6 to modulate both BER and DSB repair pathways (Tennen & Chua 2011). DSB repair by homologous recombination is reliant on the SIRT6 dependent deacetylation of C-terminal binding protein interacting protein (CtIP), which is required to resect DNA at sites of damage and facilitate repair (Kaidi *et al,* 2010). Clearly loss of this function could contribute to genomic instability which is considered a hallmark and instigator of most human cancers, including colorectal (Pino & Chung 2010).

2. SIRT6 has been shown to interact with Grap2 and cyclin-D interacting protein (GCIP), a helix-loop-helix protein and putative tumour suppressor under-expressed in a number of human tumour types, including colorectal (Ma *et al,* 2007). Loss of the positive regulatory effect of GCIP on SIRT6 could explain lower expression levels in CRC tissue and putative tumour suppressive effect.

3. SIRT6 over-expression in cancer cells initiates a massive apoptotic response resulting in attenuated proliferation of cultured cells, but not in normal cells. This response is dependent on the ADP-ribosyl transferase activity of SIRT6 through an interaction with both p53 and p73 (Van Meter *et al,* 2011).

4. SIRT6 can deacetylate H3K9 at the NF-κB promoter attenuating its transcription. Thus SIRT6 deficiency is associated with enhanced NF-κB downstream modulation of gene expression which results in enhanced resistance to apoptosis. This loss of a potential tumour suppressor mechanism could contribute to the development of neoplasia. This, however is tempered by increased levels of senescence which could compound this effect (Kawahara *et al,* 2009).

5. The role of SIRT6 in tumour cell metabolism will be discussed in due course.

Attenuated SIRT6 expression confirms previous work in our laboratory demonstrating a similar expression pattern in breast carcinoma samples compared with normal and non-malignant samples. Furthermore, SIRT6 expression proved a superior predictor of outcome when compared with current clinical scoring methods such as the Nottingham prognostic index (Zino 2010). Aside from this there is currently a lack of data placing SIRT6 expression in cancer in a clinical context. Further molecular dissection of the DNA repair

pathways involved in cancer could lead to as yet undiscovered interactions with SIRT6 and in particular its capability as a potential tumour suppressor.

5.3.1.6 Reduced relative expression of SIRT7 in colorectal cancer tissue

Predictably, studies investigating the role of SIRT7 in cancer have provided dichotomous results. Contrary to the currently presented findings of reduced SIRT7 expression in CRC tissue, SIRT7 has also been shown to be over-expressed in both breast and thyroid malignant tissue (de Nigris *et al,* 2002, Frye 2002, Ashraf *et al,* 2006). These observations could be explained by the original description of SIRT7 as a positive regulator of cell proliferation via its effect on Pol1 and apoptosis (Ford *et al,* 2006).

However, SIRT7 knockout mouse-derived MEFs display increased viability under standard and stressful culture conditions. Furthermore, tumour cells from different histological backgrounds (P19 (teratocarcinoma), NB41A3 (neuroblastoma), C3H/MCA (transformed fibroblast-derived cell line) displayed lower SIRT7 expression levels compared with non-tumour parental controls (Vakhrusheva *et al,* 2008a). The continued proliferation of SIRT7 deficient cells in the face of conditions of stress could be reminiscent of the conditions facing a cell in the early throes of neoplastic transformation. The divergence in published effect of SIRT7 expression may in part be explained by p53 status (Vakhrusheva *et al,* 2008a).

5.3.1.7 Role of the Sirtuins in Oncogenesis

It is difficult to reconcile the relationship identified between the individual sirtuins in the CRC group in this study. The demonstration of attenuated relative expression between the tumour and normal samples could indicate a mechanism whereby normal expression is suppressed in tumour cells. This could form part of a process conferring a survival advantage of tumour cells by repressing the tumour suppressive function of the sirtuins particularly SIRT1, SIRT2, SIRT3 and SIRT6. This is not inconceivable, as DBC-1 has been shown to inhibit SIRT1 activity and suppress its function (Kim *et al,* 2008, Zhao *et al,* 2008), thus a molecule up-regulated during the neoplastic process could exert a similar effect.

The combination of our demonstration of attenuated sirtuin expression with the studies mentioned above paints a clear picture of the involvement of the sirtuins in the development of cancer. However, the complexities of the up-stream regulation and down-stream targets of the sirtuins make it unlikely that a single unifying mechanism of tumour suppression (or promotion) will be demonstrated. Rather it is more likely that the effect of a particular sirtuin is context and tissue type driven. Specific examples of this would include the implication of the tumour suppressive effect in β-catenin driven colon cancer and BRCA1 associated breast cancers (Firestein *et al,* 2008, Wang *et al,* 2008a).

Recent evidence has, however, suggested one area where there could be a convergence of pathways mediated by different sirtuins, cancer cell metabolic reprogramming. Otto Warburg first demonstrated that cancer cells become reprogrammed to preferentially metabolise glucose via glycolysis irrespective of the surrounding oxygen tension. This altered pathway therefore metabolises glucose into lactate to provide ATP for the cellular energy requirements. This is termed the Warburg effect and is considered as a hallmark of a transformed malignant cell (Warburg 1956). Metabolic reprogramming requires upregulation of the enzymatic components of the glycolytic pathway such as hexokinase and phosphofructokinase, with concomitant upregulation of lactate dehydrogenase (LDH). Downregulation of the enzymes involved in the tri-carboxylic cycle (TCA) is also essential to allow the preferential metabolism of glucose to lactate (Bayley & Devilee 2012). Key recent evidence has suggested that the sirtuins, namely SIRT3 and SIRT6 may evoke a tumour suppressor effect by preventing the metabolic reprogramming of cancer cells. Finley *et al* (2011) have shown that SIRT3 can influence the cancer cell glycolytic pathway via the regulation of HIF-1, an effect mediated by mitochondrial ROS. HIF-1 is one of the main drivers of the metabolic switch, and this study demonstrates that loss of SIRT3 drives HIF-1 activation leading to a change in the gene expression profile in SIRT3 K/O mice compared with wild type littermates. In addition, treatment with the anti-oxidant N-acetylcysteine abolishes this effect, confirming the dependence on ROS. Over-expression of SIRT3 in breast cancer cell lines reduced proliferation and suppressed the Warburg effect. Furthermore, the authors show that SIRT3 protein expression is reduced in human breast cancer samples with a concomitant increase in HIF-1 and its downstream targets (Finley *et al,* 2011).

Metabolic switch to aerobic glycolysis and lactate production is one of the key features identified in the phenotype of the SIRT6 KO mouse. Furthermore this effect is also

dependent on HIF-1. Although not directly tested in tumour cells in this study, SIRT6 deficient embryonic stem cells did exhibit an increased resistance to apoptosis when exposed to hypoxia/hypoglycaemia (Zhong *et al*, 2010). However, the tumour suppressor effect of SIRT6 with particular reference to its regulation of tumour cell aerobic glycolysis has recently been characterised. In collaboration with our laboratory and through a number of experiments, Sebastian *et al* demonstrated that SIRT6 deficiency leads to tumour formation in non-transformed cells both *in vitro* and *in vivo*, with a concomitant increase in glycolysis. Furthermore, deletion of a conditional SIRT6 allele in a well characterised mouse model of colorectal adenomatous disease increases the size, aggressiveness and number of tumours. In addition, SIRT6 also plays a role in ribosome biogenesis through co-repression of MYC transcriptional activity. Finally, this study replicates our demonstration of attenuated SIRT6 expression in human cancer samples, with SIRT6 protein expression predicting both prognosis and tumour-free survival rates (Sebastian *et al*, 2012).

Thus attenuated expression of SIRT3 and SIRT6, as we have shown in CRC could provide a survival advantage for cancer cells by mediating the switch from oxidative to the preferred glycolytic metabolic pathway. Interestingly, in the correlation analysis presented in Table 5.1 SIRT3 and SIRT6 exhibit a strongly positive relationship in tumour tissue but not in normal tissue. This could indicate a common mechanism of repressed expression in the cancer cells thereby promoting cancer cell survival potentially through metabolic re-programming.

5.3.2 Sirtuin Expression & Tumour Site

The observation of SIRT2 and SIRT4 differential expression between colon and rectal cancers is intriguing, particularly when the previous demonstration of altered plasma fetuin-A levels in patients with colon and rectal cancers are considered (*Chapter 4-4.2.2.2*). A difference in the molecular pathways involved in the pathogenesis of right and left sided colon including rectal cancers is well established. Right sided cancers characteristically display features of microsatellite instability due to alteration in mismatch repair genes and variation in the CpG island methylator phenotype. Whereas, left sided and rectal cancers display evidence of chromosomal instability and accumulation of mutations

in genes implicated in the adenoma-carcinoma sequence such as adenomatous polyposis coli (APC), p53 and deleted in colorectal carcinoma (DCC) (Iacopetta 2002, Li & Lai 2009). Frattini et al report less k-ras and more APC gene mutations in rectal compared with colon cancers (Frattini *et al*, 2004). Consistent with this the APC/β-catenin pathway has been implicated in the pathogenesis of rectal cancers (Kapiteijn *et al*, 2001). This is important given the reported relationship between SIRT1 and β-catenin driven CRC (Firestein *et al*, 2008). These distinct pathways explain to some degree the differing natural history and behaviour of CRC. For example, right sided cancers are more sensitive to 5-fluorouracil based chemotherapeutic agents with a concomitant improvement in survival with adjuvant therapy (Elsaleh *et al*, 2000).

We have demonstrated that SIRT2 expression levels reduce sequentially in specimens from the right colon, left colon and rectum. This finding adds further validity to the growing body of evidence supporting the argument that CRC should in fact be classed as three entities; right colon, left colon and rectal cancer (Li & Lai 2009). Whether our observations implicate SIRT2 in either of the distinct molecular pathways contributing to colorectal cancer remains to be fully elucidated. Current data evaluating the sirtuins in this context are lacking. There are no reports of downstream regulation of any of the transcriptional factors involved in microsatellite instability by SIRT2. However, SIRT1 expression has been shown to correlate with CpG island methylator phenotype and microsatellite instability in human CRC, however no relationship with tumour site was identified (Nosho *et al*, 2009). Continued enhancement of the molecular profile of colonic and rectal cancers is particularly important when the differing treatment algorithms between the two tumour sites are considered.

5.3.2.1 Effect of Neo-adjuvant Therapy

In the current study levels of SIRT1, SIRT3 and SIRT4 were significantly increased in tumour tissue exposed to chemo-radiotherapy treatments compared with those not treated. In addition SIRT6 expression was increased within the normal samples. Perhaps of more relevance is the fact that the relationship between expression of SIRT1-4 in tumour and normal samples from patients who had undergone neo-adjuvant therapy was reversed, with SIRT1-4 expression higher in tumour samples. However, it should be borne in mind that the number of patients who had undergone neo-adjuvant therapy in the group were small (n=6) and as such this data should be regarded as preliminary and interpreted with caution.

It should also be emphasised that only patients with rectal cancer undergo neo-adjuvant treatment for the reasons outlined in the introduction to this chapter.

The finding of sirtuin up-regulation in samples of both tumour and normal rectal tissue from patients who had undergone neo-adjuvant therapy is logical when the function, particularly of SIRT1 is considered. The anti-proliferative effects of chemo/radiotherapy are mediated through DNA damage and subsequent induction of senescence or apoptosis (Begg *et al,* 2011). SIRT1 has been shown to play a role in the stress and DNA damage response induced by ionizing radiation, a feature that has led investigators to conclude that aberrant SIRT1 expression may in part modulate tumour cell resistance to chemo/radiotherapy (Olmos *et al*, 2011). Indeed, much of the work pertaining to the *in vivo* expression of SIRT1 and the response to chemo/radiotherapy has been performed in the context of treatment resistant tumours. Chu *et al* (2005), analysed expression of SIRT1 in cancer specimens from patients who had not responded to chemotherapeutic treatment. In these chemo-resistant patients SIRT1 levels as detected by western blot analysis were found to be significantly increased in tumour tissue after chemotherapy treatment and were associated with expression of the multidrug resistant molecule P-glycoprotein (Chu *et al*, 2005). The small number of patients who had undergone neo-adjuvant treatment and a lack of clinical data related to treatment regimes prevented a more in depth analysis of resistance to treatment in the current CRC group. However, the finding of differential expression of SIRT1 could indicate a potential role of SIRT1 in the tumour cell response to chemotherapy.

Data pertaining to the remainder of the sirtuins and the response to chemotherapy are sparse. In the Chu *et al* study mentioned above none of the remaining sirtuins were differentially expressed in response to chemotherapy (Chu *et al,* 2005). SIRT3 has been shown to translocate to the mitochondria in cells treated with etoposide or UV irradiated, an indication of SIRT3 involvement in the stress response (Scher *et al,* 2007). Meanwhile, *SIRT6*-null mouse cells are hypersensitive to DNA damage by ionizing radiation (Mostoslavsky *et al,* 2006). These findings could in part explain the finding of augmented levels of SIRT3 and SIRT6 in tumour specimens from patients exposed to chemo-radiotherapy.

Drawing firm conclusions regarding the interaction between the sirtuins and the cellular response to chemo-radiotherapy is difficult using the data presented in this chapter. Clearly

further characterisation in larger numbers of therapy exposed tissue with relevant controls is required. It would also be interesting to gain further information regarding sirtuin expression and the pathological response to treatment as manipulation of sirtuins expression could therefore be used to improve tissue responsiveness to neo-adjuvant treatment.

5.3.3 Sirtuin Expression as a Marker of Prognosis

Correlation of our sirtuin expression profile with pathological parameters has demonstrated a significant association between SIRT4 and node positive disease, with SIRT4 expression higher in node positive disease. Furthermore, the group of patients with higher SIRT4 expression also had poorer survival. Currently, patients who are deemed to have more aggressive disease determined by either nodal status or adverse pathological features such as those quantified by the Peterson Index are offered adjuvant chemotherapy, co-morbidity permitting (Petersen *et al,* 2002). In node positive disease adjuvant therapy results in a survival gain of 10-15%, however the benefits in node negative disease are much less clear with a survival gain of only 3.6% (Cunningham *et al,* 2010). We have previously demonstrated increased SIRT3 in node positive breast cancer patients (Ashraf *et al,* 2006), whilst Cha et al have shown SIRT1 protein expression to correlate with nodal status and other markers of disease severity, as well as being a significant prognostic indicator in gastric carcinoma (Cha *et al,* 2009). Attenuated SIRT2 expression identified using gene expression array analysis has also been proposed as a poor prognostic indicator in oesophago-gastric carcinoma (Peters *et al*, 2010).

The relevance of the relationship between SIRT4, nodal status and survival is not entirely clear, as although SIRT4 has well recognised effects on metabolism and response to CR in mice (Haigis *et al,* 2006), it has not been shown to have any direct effect on the oncogenic process. The preponderance of deaths in the group of patients with higher SIRT4 expression is almost certainly as a result of more aggressive disease as indicated by nodal status. It should be borne in mind that there were relatively few deaths in the high SIRT4 expression group with none in the low expression group, meaning this could be an anomaly. Increasing the study numbers could help to fully delineate this relationship. Further work to fully characterise SIRT4 post-translational expression levels in cancer specimens will determine whether it may be used as a novel molecular marker of risk

stratification or predictor of outcome, which could be used to select patients who may benefit from adjuvant therapy, or be used as a pathological marker of severity.

The demonstrated difference in SIRT4 expression with stage of disease could indicate that it is not only the level of expression but the timing of this response that is important in the oncogenic process. SIRT1 expression has been shown to sequentially decrease from normal tissue, through non-malignant adenomas to high grade malignant disease (Kabra *et al,* 2009). This could indicate that SIRT1 levels are maintained at a high level in normal tissue and pre-cancerous lesions to prevent malignant transformation. The exact stimulus for this change in expression can only be speculated upon but could be related to cellular stress levels and the subsequent activation of downstream damage response pathways. The adenoma-carcinoma multi-hit sequence of events in colorectal oncogenesis could be particularly important in this scenario as mutations of genes such as p53, APC and k-RAS could interact and alter sirtuin expression with obvious downstream effects. In this study it is not clear if this is the reason for the noted differential expression of SIRT4. Within the tumour cells SIRT4 expression could be upregulated from its suppressed state to allow to the tumour to continue to proliferate indicated by advanced stage. The data pertaining to the remainder of the sirtuins and the relationship with tumour stage i.e. similar expression levels throughout stages could indicate the sirtuins are important for initiation and development of CRC, not just at the later stages.

5.3.4 Inter-Relationships between Sirtuins

Using the generated expression profile for the individual sirtuins association analysis was performed to determine any significant inter-relationships. This analysis yielded multiple significant associations between individual sirtuins. Whilst it is difficult to speculate upon the exact significance of these findings a number of general conclusions can be drawn. A striking feature is that all the associations deemed significant by statistical analysis were of a positive nature, where for example as SIRT1 levels increase so do SIRT2, SIRT3 and SIRT4 (Table 5.1), in a highly significant relationship. Within the tumour samples the relationship was most prominent with SIRT2, SIRT3 and SIRT4 in whom there was a highly significant relationship with all but one of the other sirtuins (Table 5.1). These data could indicate a common regulator of sirtuin expression responsible for controlling the transcriptional response to appropriate stimuli.

Whilst some of the individual downstream regulators of individual sirtuins are mentioned above, common control pathways between the sirtuins have been difficult to identify, if indeed they do exist. The most obvious ubiquitous control mechanism for sirtuin activity is the dependence by the sirtuins on the metabolic intermediary NAD^+ as a cofactor for activity. As mentioned earlier this introduces the potential for nutritional manipulation to alter sirtuin activity. Further layers of expressional control are conferred by some of key effectors of the cellular stress response, SIRT1 and SIRT2 have both been shown to be transcriptionally up-regulated by the acetyl transferase p300 with concomitant effects on p53 activity (Han *et al,* 2008b).

Table 5.1 shows a clear correlative relationship between the mitochondrial sirtuins (SIRT3, SIRT4 and SIRT5) in the tumour samples. This could indicate commonality of transcriptional control and function, however no such relationship has been previously identified. As mentioned above SIRT3 has been recently implicated in the metabolic reprogramming essential for tumour cell proliferation (Finley *et al,* 2011). The demonstrated correlation between the mitochondrial sirtuins in our CRC tumour samples could implicate their involvement in this process. Very little is known regarding common pathways involving the mitochondrial sirtuins. However, Nakamura *et al* (2008) have demonstrated that intra-cellular shuttling of SIRT3 is dependent of co-expression with SIRT5 (Nakamura *et al,* 2008). This raises the possibility SIRT5 may not possess a tumour suppressor function *per se* but it is required in some capacity for SIRT3 to function, indicated by their demonstrated relationship in CRC tumour cells.

A common expression mechanism between the sirtuins could be responsible for the positive inter-associations demonstrated, however, given the complexities of sirtuin regulation and functionality it seems unlikely that such a simple control mechanism would be utilised.

5.3.5 Sirtuin Expression and Biological Age

Our data demonstrate a link between low sirtuin expression levels and systemic inflammation. Sirtuin expression and control of the inflammatory cascade are intricately linked through the control of NF-κB transcription by SIRT1 (Yeung *et al,* 2004) and SIRT6 (Kawahara *et al,* 2009). This relationship has been investigated in a clinical context

by Singh et al who have reported that SIRT1 is implicated in the pathogenesis of colitis by inversely regulating NF-κB and that inducing SIRT1 activity by resveratrol abrogates the process in a well characterised mouse model of colitis (Singh *et al,* 2010). Lower SIRT1 levels have also been reported in the lungs and macrophages of patients with COPD (Rajendrasozhan *et al,* 2008). The presence of a systemic inflammatory response in cancer patients confers poor survival independent of factors such as tumour stage. This systemic inflammation is thought to represent a host characteristic (Roxburgh & McMillan 2010). The association between altered sirtuin expression in normal colorectal tissue and indices of systemic inflammation could implicate the sirtuins in this host response. The sirtuin expression profile namely suppressed SIRT2, SIRT3, SIRT4 and SIRT7 could highlight a previously undiscovered role in the control of pro-inflammatory pathways for these genes, an effect which requires validation in *in vivo* models.

We have observed, in the context of low expression in tumour samples a significant inverse relationship between SIRT3 and TL. This finding is a pertinent one given that short TL indicates advanced biological age of these cells, as a result of uncontrolled cell turnover. Higher SIRT3 expression levels in this scenario could be a response to dysregulated mitochondrial function evident in these biologically aged neoplastic cells. This association between sirtuin expression and TL lends further support to our hypothesis that the sirtuins link TL and hence DNA damage response with energy utilisation via the MTR.

5.3.6 Conclusion

The expression data presented above provides clear evidence that sirtuin expression is attenuated in the colorectal cancer specimens under investigation, indicating a tumour suppressive effect. In order to lose the tumour suppressive effect with the result of oncogenic transformation of a colorectal epithelial cell to a malignant cancer cell, sirtuin expression must be repressed to result in the attenuated expression levels we have demonstrated. As with many aspects of the sirtuin story this is almost certainly a process with a great variety of complicating factors not only dependant on the molecular characteristics of tumour type but factors closely associated with the sirtuins themselves. Namely, the up-stream regulatory mechanisms which can control expression, the sub-

cellular localisation and the presence or otherwise of sirtuin isoforms may complicate interpretation.

Information regarding the regulation of sirtuin expression is scanty with much more known about their functional downstream targets. A number of mediators act at the promoter level to repress SIRT1 transcription. Both HIC-1 (Chen *et al,* 2005a) and miR34-a (Yamakuchi & Lowenstein 2009) exert a tumour suppressive effect via feedback loops in a p53 dependant fashion to reduce SIRT1 transcription. This relationship could be of particular importance as recent evidence suggests that levels of miR34a increase relative to chronological age with a reciprocal attenuation in SIRT1 protein levels (Li *et al,* 2011b). Thus age related reduction in SIRT1 expression through a miR34a dependent mechanism would mean a loss of its tumour suppressor effect and could also partly explain the increasing incidence of cancer with chronological age. One potential complicating problem is the presence or absence of mutated p53.

Other mechanisms of suppressed sirtuin gene expression can only be speculated upon. Environmental factors are known to affect sirtuin expression, the most famous example being the induction of sirtuin expression in particular SIRT1, SIRT3 and SIRT6 by calorie restriction (Cohen *et al,* 2004b, Kanfi *et al,* 2008b, Qiu *et al,* 2010). As mentioned previously calorie restriction has consistently been shown to increase lifespan in a number of different model systems. In addition, it has also been shown to reduce the incidence of cancer in higher organisms (Omodei & Fontana 2011). This could imply that the tumour suppressor effect of calorie restriction is in part mediated through induction of sirtuin expression. Conversely, a diet high in fat has been shown to induce tumour progression and features of metastasis in various animal models of colon cancer (Park *et al,* 2011, Tang *et al,* 2012). Furthermore, recent studies have indicated this may be mediated through induction of β-catenin targets regulating colon epithelial cells and Wnt signalling pathway (Liu *et al,* 2011, Padidar *et al,* 2012). Whilst sirtuin modulation has not been shown to be directly affected by high fat diet in animal models of cancer, suppression of SIRT1 function has been demonstrated in atherosclerotic lesions. In animals fed a high fat diet p53 acetylation at K382 (the acetylation site specific to SIRT1) was significantly reduced indicating this dietary regimen could influence sirtuin expression (Xu *et al,* 2011). Similarly, mice fed a high fat diet exhibit attenuated hepatic SIRT3 expression and activity (Kendrick *et al,* 2011). It is enticing to think that diet could suppress sirtuin expression to

the extent that the tumour suppressive capacity would be lost in a tissue specific context contributing to the oncogenic process.

It is clear there is a dearth of knowledge regarding the controlling mechanisms of sirtuin expression. Other complicating factors which could influence not only sirtuin expression but activity are;

- Epigenetic phenomena such as silencing of the respective sirtuin genes, could alter their expression through chromatin remodelling. As yet there are no reports in the literature either *in vitro* or *in vivo.*

- Post-translational modification such as phosphorylation at specific residues within the SIRT1 enzyme can alter activity. Indeed, cyclinB/Cdk1 has been identified as a key regulator of SIRT1 phosphorylation (Sasaki *et al,* 2008). In addition, a recent key study by Back *et al* (2011), demonstrated that phosphorylation of SIRT1 by mTOR promotes prematurely senescent squamous cell carcinoma cell survival. This effect was seen in the absence of functional p53 and could re-constitute the scenario of human *in vivo* cancer recurrence where tumour cells re-enter the cell cycle causing recurrent disease (Back *et al,* 2011).

The effect of the sub-cellular localisation of the sirtuins on their function and potential tumour suppressor role has not been established. The basic pattern of sub-cellular localisation is well established, however evidence is emerging that there are some caveats to this picture. Byles *et al* (2010) have demonstrated that SIRT1, which was previously thought to be predominantly nuclear in location, is present in the cytoplasm of DU-145 (prostate cancer) cells. This occurs as result of increased protein stability and is regulated by elevated mitotic activity (Byles *et al,* 2010). Whilst, SIRT2 has previously been shown to shuttle between the nucleus and cytoplasm depending on the stage of the cell cycle (North & Verdin 2007a). These studies demonstrate the dynamic nature of sirtuin sub-cellular localisation, it seems likely that the localisation is dependent on tissue type and prevailing cellular conditions. This phenomenon has already been demonstrated with regards to SIRT3 which has been shown to shuttle from the nucleus to mitochondria on sensing of cellular stress (Scher *et al,* 2007).

Clearly there is ambiguity in the literature as to whether the sirtuins, in particularly SIRT1 are tumour suppressive or oncogenic. This is reflected in studies which have attempted to

pharmacologically manipulate sirtuin activity to alter the oncogenic process. The sirtuin modulator which has garnered the most publicity is the polyphenol resveratrol. Whilst several anti-cancer properties have been related to resveratrol there is considerable controversy in the scientific literature regarding the SIRT1 dependency of its mechanism of action. Claims and counter claims argue that SIRT1 is activated downstream of AMPK which is initially activated by resveratrol due to its ability to inhibit phosphodiesterases thereby increasing cAMP availability. The overall effect is an increase in NAD^+ levels leading to SIRT1 activation (Chung 2012, Park *et al*, 2012). Nonetheless, trials are underway to determine whether treatment of patients with resveratrol can recapitulate the chemo-preventative effects seen *in vitro* and in murine models. In this regard resveratrol has been shown to be safe in the quantity required to produce a biological effect *in vivo*. Furthermore, resveratrol also caused a modest reduction in cell proliferation in CRC tissue samples. Unfortunately this study did not examine any of the purported downstream targets of resveratrol (Patel *et al*, 2010). Pharmaceutical companies have sought to utilise the beneficial effects of SIRT1 activation by developing activating compounds (STACs). A number of these have been developed, one such compound, ST1720 has been shown to reduce the proliferation of multiple myeloma cells *in vitro* via SIRT1 dependent apoptosis (Chauhan *et al*, 2011).

Whilst our data support the notion that activation or upregulation of SIRT1 could produce beneficial effects, it is not clear what effect this may have on the rest of the sirtuins. The demonstration of correlation between sirtuin expression patterns could theoretically indicate that activation of SIRT1 may influence the expression of the remaining sirtuins. However, despite a number of studies investigating the effect of pharmacological SIRT1 activation none have examined the effect of activation of any of the other sirtuins or what the effect of SIRT1 activation has on downstream SIRT2-7 levels. As indicated there are clear mechanistic pathways by which the remainder of the sirtuins affect the oncogenic process with particularly strong evidence supporting the tumour suppressive effect of SIRT3 and SIRT6. It may be pharmacological augmentation of SIRT responsiveness could alter the oncogenic process at various stages. For example increasing SIRT6 expression specifically in colorectal adenomas could promote genetic stability and prevent onward progression through the adenoma-carcinoma sequence.

In summary, we have created a unique expression profile for SIRT1-7 in colorectal cancer patients. This has provided clear evidence that expression of all seven sirtuins is reduced in

cancer specimens when compared with normal tissue. These findings support the argument that sirtuins act as tumour suppressors in colorectal cancer. However, the in vivo role of the sirtuins in oncogenesis is likely to be complex. Further work is required to fully delineate whether there is molecular ‘cross-talk’ between individual sirtuins and whether their role will be dependent on tumour type and distinct molecular pathways driving oncogenesis. Our analysis has highlighted novel associations linking sirtuin expression with factors indicative of biological ageing namely inflammation and TL. Furthermore we have utilised sirtuin expression to differentiate between tumour site and pathologically more aggressive disease. Thus study providing clear evidence that targeting of individual sirtuins could prove useful in the quest to identify novel chemotherapeutic agents.

CHAPTER 6: GENERAL DISCUSSION

The data presented in this thesis supports the original hypothesis of biological ageing contributing to the pathogenesis of CRC in the study population. This relationship has been demonstrated by aberrancy of two molecular pathways shown to influence biological ageing namely accelerated telomere attrition and altered sirtuin expression.

6.1 Miles on the Clock Hypothesis

The paradigm whereby altered telomere biology could lead to the development of cancer is intuitive, with the multi-factorial determination of TL and rate of attrition a key concept. Whilst there was no significant relationship between socio-economic status and TL in the group under investigation in this study, the well documented pro-inflammatory and oxidative state found in this population is likely to reflect chronic exposure to a variety of environmental and lifestyle stressors, for example cigarette smoke and poor diet. The level of socio-economic deprivation in the study population may of course confound results by not giving an accurate representation of the population in general but it does provide an ideal environment to test the proposed hypothesis. A pro-inflammatory and oxidative state could impact on telomere attrition rates in a number of ways. Clearly increased inflammatory cell turnover would lead to progressive telomere attrition in the haemopoietic compartment. This progressive telomere attrition could then lead to a state of crisis and senescence with subsequent release of pro-inflammatory mediators compounding the pro-inflammatory state. Low socio-economic status is typified by a poor quality, high fat content and sugar rich diet. This has been shown to result in enhanced production of ROS resulting in a pro-oxidative state. As described in depth previously ROS are directly toxic to telomeric DNA resulting in acceleration of telomere attrition. This pro-inflammatory state and cumulative oxidative burden is therefore thought to be key to the acceleration in biological ageing and increased cancer risk in the current study group. Indeed socio-economic deprivation such as that seen in the cancer patients under investigation has recently been associated with accelerated telomere attrition (Shiels *et al*, 2011). This state in which patients display evidence of biological ageing in the form of progressive telomere attrition has been equated colloquially with the concept of a car running up miles on the clock, hence this has been termed the ‘miles on the clock’ hypothesis. Generalised telomere dysfunction could then impact on cancer risk and

progression as outlined previously by promoting chromosomal instability and altering the peri-tumoral milieu via cellular senescence.

6.2 MTR Hypothesis

As described earlier the sirtuins provide an intuitive link between the energy dependent sensing of DNA damage and initiating the optimal response of the cell to either repair the damage or prevent replication of potential mutations by facilitating either apoptosis or senescence. Optimal responsiveness of the MTR trinity would require normal functioning of each of the triumvirate as well as fine control of sirtuin expression. Alteration in any of these components could lead to knock-on effects on the remainder of the pathway. It is appealing to speculate that aberrancy of this pathway could play a role in biological ageing and the development of some of the pathological processes associated with it, particularly cancer.

The results presented in this thesis provide clear and compelling evidence that accelerated telomere attrition and attenuated sirtuin expression are implicated in the pathogenesis of CRC. Aberrancy of these pathways supports the hypothesis that dysregulation of the MTR could play a role in CRC development. Interplay between elements of the MTR is evident with the identified association between SIRT3 expression and tumour tissue TL. As mentioned earlier elevated SIRT3 expression could be a response to dysregulated mitochondrial function in neoplastic cells or could indicate a novel functional relationship between SIRT3 and telomere biology required for uncontrolled cellular proliferation. Such a relationship has recently been identified in hepatocellular carcinoma where SIRT1 was shown to be involved in telomere maintenance (Chen *et al*, 2011).

6.3 Clinical Translation

Utilisation of some of the aspects of biological ageing investigated in this thesis could prove useful in the continued quest to treat cancer of different histological subtypes and at various interventional stages.

6.3.1 Epidemiology

The concept of biological ageing as a risk factor for cancer is an attractive proposition as it opens the possibility of identifying a window of opportunity where through lifestyle modification the process of biological ageing could be retarded thereby reducing cancer risk. Possibilities to achieve this could be through further rationalisation of the determinants of TL or identification of new contributing factors, such as the potential interaction with fetuin-A identified in this study. Ornish *et al* (2008), report that comprehensive lifestyle modifications can increase telomere maintenance mechanisms in the white cells of patients with prostate cancer (Ornish *et al*, 2008). This study provides preliminary evidence that lifestyle modification could alter the process of biological ageing. Further support for the ability of lifestyle modification to alter the course of the bio-ageing process comes from a recent study by Du *et al* (2012). The authors report a modestly significant association between self-reported activity levels and TL, with increasing levels of activity associate with longer TL after adjustment for confounding factors such as age and BMI (Du *et al*, 2012). If confirmed in larger population based randomised controlled trials these data would support the more intensive adoption of public health measures to improve cancer risk through lifestyle modification.

6.3.2 Diagnosis & Prognosis of Cancer

This study has demonstrated an association between aspects of biological ageing (PBL telomere length and SIRT4 expression) and pathologically more aggressive disease. Stratifying patients using molecular markers of severity could allow the identification of patients who may benefit from a more aggressive management strategy which could in turn facilitate overall improvements in outcome. Clearly more work is required to validate these molecular markers in large numbers of patients and potentially in different types of cancer. The ability to differentiate between anatomical tumour sites in this group of CRC patients using factors involved in biological ageing (fetuin-A, SIRT2 and SIRT4) supports further work to improve the molecular characterisation of these tumours. These differences could be exploited to propose different management strategies for CRC depending not only on anatomical tumour site as is currently the case but also in conjunction with molecular differences.

6.3.3 Therapeutic targets

One of the defining aims of this study is to provide further information of the expression of ageing related genes in CRC with the aim of identifying potential targets for intervention. The demonstration of attenuated sirtuin expression in CRC raises the possibility that enhancing sirtuin signalling could provide a tumour suppressor effect. The difficulty with this lies in the fact that depending on the cancer type and model used sirtuin expression has opposing effects. It is likely, therefore, that a 'one size fits all' approach to developing agents which could alter sirtuin activity will not yield the desired results. Hence an approach using the concept of personalised chemotherapy where cancers are targeted on an individual basis depending on the genetic and epigenetic profile could prove useful. In the case of the sirtuins determination of p53 status, acetylation status, molecular driver of the cancer e.g. β-catenin or the recognition of polymorphic forms of the sirtuin genes could dramatically affect the activity of proposed agents. Further refinement may also be possible using techniques such as RNA interference to target and deliver specific genes. The era of personalised chemotherapy in clinical practice is in its infancy, however KRAS mutation testing and subsequent intervention using anti-EGFR monoclonal antibodies is now a management option in metastatic CRC (Van Schaeybroeck *et al*, 2011). The finding of increased sirtuin expression in patients who had undergone neo-adjuvant treatment indicates a potential role for the sirtuins in the response to chemo-radiotherapy. It may therefore be possible to exploit this to allow more efficient delivery and hence improve response to current treatment regimes.

6.4 Limitations & Future Work

A constant theme through the telomere and sirtuin components of this thesis has been the contribution played by p53 to both of these pathways. Therefore, determination of the p53 status of the tumours and also patient p53 genotyping would allow a more comprehensive approach to elucidating the interplay between the biological aging and cancer pathways. Similarly, relating SIRT1 expression to the numerous other non-histone proteins such as FOXO, NF-κB or Bcl, over which it has been shown to influence would provide greater understanding of the mechanistic pathways involved in the cancer model used in this investigation.

With regards to the investigation into the contribution of telomere attrition in CRC the main area in which could be improved is to have a completely matched group with blood cells and tumour tissue from the same patient. Increasing the overall numbers would also enhance the capability to tease out relationships between TL and patient sub-groups which were not identified during analysis of the current data. Identifying further clinically important relationships could improve the therapeutic potential of telomere biology in the cancer setting. It may also be possible that the methods used to delineate any relationship between TL and oxidant status were not sensitive enough. Methods to detect fine changes in ROS or their intermediaries at the sub-cellular level may provide a more accurate representation of the flux in oxidative potential and the effect on TL.

To gain a more accurate representation of sirtuin expression incorporation of a non-malignant group from a patient source with no history of cancer with which to compare sirtuin relative expression levels from the cancer and adjacent normal tissue would have been useful. This further comparison would have negated any potential effect of a field change in sirtuin gene expression which extended from the peri-tumoural area to adjacent normal tissue. Incorporation of such a group was however, not possible through the local Biobank repository for ethical reasons. Further improvements in the overall picture of sirtuin expression would be to quantify protein expression using western blot analysis. Comparing the current mRNA expression profile with the protein expression profile could also determine the presence and significance of post-translational modification. In addition, immunohistochemical staining of tissue arrays could address the issue of sub-cellular localisation of the sirtuins in cancer cells. As mentioned in the relevant discussion section pre-neoadjuvant treatment biopsies would have allowed a more accurate conclusion to be drawn as to the effect on sirtuin expression by this treatment. This could be complemented by *in vitro* analysis of cultured cells exposed the chemo-radiation. Recent evidence has also suggested that variation in SIRT1 genotype may have an effect on its clinical effect (Zillikens *et al*, 2009). Whilst this was not considered in this thesis a more refined picture of sirtuin expression may be possible by examining the effect of sirtuin genotype in cancer patients.

6.5 Final Conclusion

The results reported in this thesis support the initial hypothesis of biological ageing playing a role in the pathogenesis of CRC in our patient group. We have observed that patients with CRC have significantly shorter telomeres than control subjects, congruent with accelerated biological ageing in the pathogenesis of CRC. These observations are in keeping with the hypothesis of telomere attrition predisposing to disease. Furthermore, patients with shorter telomeres display evidence of systemic inflammation and pathologically more advanced disease. An imbalance in redox control mechanisms and calcium homeostasis may be a contributing factor to telomere dynamics in our group of patients. Further refinement of the factors determining TL and subsequent manipulation could alter the risk profile of CRC.

Furthermore, we have created a unique expression profile for the ageing associated genes SIRT1-7 in CRC patients. We have provided clear evidence that expression of all seven sirtuins is attenuated in cancer specimens when compared with normal tissue, lending further support to the argument that sirtuins act as tumour suppressors in colorectal cancer. However, the in vivo role of the sirtuins in oncogenesis is likely to be complex with molecular ‘cross-talk’ between individual sirtuins, tumour type and the molecular profile of an individual tumour all important factors in determining the overall effect of the individual sirtuins. This analysis has highlighted novel associations linking sirtuin expression with factors indicative of biological ageing namely inflammation and TL. Furthermore sirtuin expression proved a useful molecular mechanism to differentiate between tumour site and pathologically more aggressive disease. This study provides clear evidence that targeting of individual sirtuins could prove useful in the quest to identify novel chemotherapeutic agents.

Appendix 1

β-actin Fwd - GGTCACCCACACTGTGCCCAT
β-actin Rev – GGATGCCACAGGACTCCATGC

Gene	*Fwd Primer 5'-3'*	*Rev Primer 3'-5'*	*Taqman™ probe*
SIRT1	TAGAGCCTCACATGCAAGCTCTA	GCCAATCATAAGATGTTGCTGAAC	ACTCCAAGGCCACGGATAGGTCCATATACTT
SIRT2	CCTCGCCTGCTCATCAACA	TCCTCCGAGGCCCATAATC	TGGCCAGTCGGACCCTTTCCTG
SIRT3	CATTCGGGCTGACGTGATG	AACCACATGCAGCAAGAACCT	TGCACCGGCGTTGTGAAGCC
SIRT7	CGTCCGGAACGCCAAATAC	GACGCTGCCGTGCTGATT	TGGTCGTCTACACAGGC

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