Biological Ageing and Colorectal Cancer: Fetuin A, Sirtuins and Telomeres at the Interface between Inflammation, Metabolism and Cancer

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Abstract

Colorectal cancer is a common, age-associated disease with significant co-morbidity and mortality. Biomarkers of ageing may have prognostic or predictive value in colorectal cancer. Fetuin A, members of the sirtuin family of proteins and telomeres have shown promise as potential biomarkers of ageing.

AIM: To evaluate these potential biomarkers in the context of colorectal cancer.

METHODS: Two cohorts of patients were used. Telomere length was measured in peripheral blood leukocytes (PBL), and for a subset of patients, in normal colorectal and colorectal tumour tissue. Serum fetuin A was measured for these patients and data on clinico-pathological factors of accepted significance in colorectal cancer was collected prospectively. Telomere length in the matched samples of leukocytes, normal colorectal and colorectal tumour tissue was compared. Associations between telomere length in the different tissues, serum fetuin A and clinico-pathological factors of accepted significance in colorectal cancer were evaluated. A systematic review of the literature was performed to examine the evidence for correlation between telomere length in different tissues in humans.

Tissue from colorectal tumours from the second cohort of patients was mounted in a tissue microarray (TMA) and stained for sirtuin proteins (SIRT2-SIRT7). This TMA also contained tissue from a subset of matched samples of adjacent normal colorectal mucosa. Staining of normal colorectal and colorectal tumour tissue was evaluated by the weighted Histoscore method and compared. The effect of staining in tumour tissue on cancer-specific survival was examined. Associations between Histoscores and clinico-pathological factors of accepted significance in colorectal cancer were assessed.

RESULTS: Systematic review of the literature did not show robust evidence of correlation between telomere length in different tissues in humans. Telomere length in peripheral blood leukocytes did not show correlation with telomere length in normal colorectal mucosa, or in colorectal tumour tissue.

PBL telomere length was potentially related to the presence of distant metastases. Fetuin A was inversely associated with markers of systemic inflammation and with T stage.

Novel nuclear localisation was described for SIRT4 and SIRT5. Protein expression of the sirtuins was reduced in tumour tissue in comparison to normal colorectal mucosa, apart from SIRT3 cytoplasmic and nuclear and SIRT6 nuclear stainng. Lowest and highest quartile SIRT2 expression was associated with worse survival.

Sirtuin protein expression levels and localisation correlate with increased systemic inflammation and pathological markers of poor prognosis in tumour tissue.

Intercorrelations between sirtuin expression levels in normal tissue are not seen in tumour tissue, possibly indicating a breakdown of signalling and control.

DISCUSSION: These results indicate that PBL telomere length cannot be used as a surrogate measure for telomere length in the colorectal mucosa, nor by extrapolation, provide a measure of the biological age of this compartment. However, individualised measures of PBL telomere length may be useful in assessing frailty. Colorectal tissue telomere length may be useful for screening or surveillance of this compartment, but requires further evaluation. Fetuin A may be a useful marker of local invasion, but it is not clear whether this offers information independent of inflammatory status.

Expression of SIRT2 in the nucleus requires further assessment as a poor prognostic marker in colorectal cancer. The information available on the function of sirtuins in colorectal tissue is limited. Novel subcellular localisations, particularly for SIRT3, SIRT4 and SIRT5 in the nucleus and for SIRT6 in the cytoplasm described here will need to be verified in normal tissue. There are significant alterations in sirtuin protein expression in tumour tissue with respect to normal tissue. These, with the correlations with markers of systemic inflammatory status and pathological indicators of poor prognosis reinforce the important place of the sirtuins in colorectal cancer biology. A precise understanding of the pathways involved could open up new avenues of research

and therapeutic potential using the diverse functions of this important family of proteins to the patient's advantage once again.

Network analysis of interactions within the human proteasome is a growing area of research and is likely to be of particular use in the further evaluation of each of the multifunctional markers of cellular resilience investigated in this thesis.

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Albert Einstein

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Go raibh mile maith agaibh!

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: _____

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Abbreviations

5-FU	5-fluorouracil
AASIS	amino acid stimulated insulin secretion
AceCS	Acetyl CoA synthase
ACPGBI	Association of Coloproctology for Great Britain and Ireland
ADP	adenosine diphosphate
AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B
ALDH2	Aldehyde dehydrogenase 2
ALT	alternative lengthening of telomeres
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase or 5' adenosine monophosphate-activated protein kinase
APC	adenomatous polyposis coli
APC/C	Anaphase-Promoting Complex, cyclosome
APE1	Apurinic/ apyrimidinic endonuclease-1
APPs	acute phase proteins
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATP5O	ATP synthase subunit O-mitochondrial
BER	base excision repair
BMP	bone morphogenetic proteins
BNP	B-type natriuretic peptide
BSG	British Society of Gastroenterology
COPD	chronic obstructive pulmonary disease
COX-1/2	cyclo-oxygenase 1 or 2
CPS1	carbamoyl phosphate synthetase 1
CR	caloric restriction
CRC	colorectal cancer
CRP	C-reactive protein
CSF	cerebrospinal fluid
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
DPX	di-n-butyl-phthalate in xylene
DSB	double strand break
dsDNA	double-stranded DNA
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-to-mesenchymal transition
eNOS	endothelial nitric oxide synthase
ER1	oestrogen receptor 1
ERK	extracellular-signal-regulated kinases, specific subset of the mammalian mitogen-activated protein kinase family
ESC	embryonic stem cell
FAP	familial adenomatous polyposis
FGF2	fibroblast growth factor 2
FISH	fluorescence in-situ hybridization
GDH	glutamate dehydrogenase
GFP	green fluorescent protein
GLUT-1	glucose-transporter-1
GLUT-4	glucose-transporter-4
GSK	glycogen synthase kinase
H+E	haematoxylin and eosin

HCC	hepatocellular carcinoma
HIC1	Hypermethylated-in-cancer protein
HIF-1α, HIF-2α	Hypoxia-inducible factor 1-alpha, 2-alpha
HNF-3	hepatocyte nuclear factor 3
HNPCC	hereditary non-polyposis colorectal cancer
HRAS	transforming protein p21, one of the RAS family of oncogenes
HRP	horseradish peroxidase
HUVECs	human umbilical vein endothelial cells
IBD	inflammatory bowel disease
ICAM1	intercellular adhesion molecule 1
IGF	insulin–like growth factor
IHC	immunohistochemistry
IHF	ischaemic heart failure
IL1β	interleukin-1 beta
IL6	interleukin-6
IL8	interleukin-8
IL10	interleukin-10
IPF	idiopathic pulmonary fibrosis
IR	infra-red (radiation)
IUGR	intrauterine growth restriction
KRAS	Kirsten rat sarcoma viral oncogene homolog, one of the RAS family of oncogenes
KRT23	Keratin, type I cytoskeletal 23 protein
LDH	lactate dehydrogenase
LPS	lipopolysaccharide (endotoxin)
LSAB	labelled streptavidin-biotin
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
MCT1	monocarboxylate transporter 1
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor
MEK	mitogen/extracellular signal-regulated kinase, mitogen-activated protein kinase kinase
MeSH	Medical subject heading
mGPS	modified Glasgow Prognostic Score
MMP	matrix metalloproteinase
MMS	methylmethane sulphonate
MnSOD	manganese superoxide dismutase
MRN complex	MRE11/RAD50/NBS1 complex
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAMPT/PBEF	nicotinamide phosphoribosyltransferase, pre-B cell colony-enhancing factor
NBS1	Nijmegen breakage syndrome 1 protein
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end-joining
NHS	National Health Service
NICD	Notch1 intracellular domain

NMN	nicotinamide mononucleotide
NMNAT	nicotinamide/nicotinic acid mononucleotide adenylyltransferase
NSAIDs	non-steroidal anti-inflammatory agents/drugs
NTC/ntc	no template control
NTF	no tumour found
OOADPr	2′-O-acetyl-ADP-ribose, product of sirtuin deacetylation
PAI1	plasminogen activator inhibitor-1
PAMPs	pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerase
PAS kinase	PAS domain-containing serine/threonine-protein kinase
PBL	peripheral blood leukocyte
PBMC	peripheral blood mononuclear cells
PCAF	transcriptional coactivator associated with p53
PCR	polymerase chain reaction
PDK	pyruvate dehydrogenase kinase
PEPCK1	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1-alpha
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinases
PK	pyruvate kinase
PKA	protein kinase A
PMN	polymorphonuclear leukocytes
POT1	protector of telomeres 1
PPARγ	peroxisome proliferator-activated receptor gamma
PPP	pentose phosphate pathway
PRMs	pattern recognition molecules
PSA	prostate specific antigen
PTEN	tumour suppressor regulating PI3K/AKT pathway
PTP	permeability transition pore
PVD	peripheral vascular disease
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RAF	one of 3 serine/threonine-specific protein kinases that are related to retroviral oncogenes
RAP1	repressor/activator protein 1
rDNA	ribosomal DNA
REC	Research and Ethics Committee
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT-PCR	real-time or quantitative PCR
SC	standard calibrator
SD	standard dilution
SDHA	succinate dehydrogenase A
SHC	src homology 2-domain-containing protein
shRNa	small hairpin RNA
siRNA	Small interfering RNA, short interfering RNA or silencing RNA
SIRT1, SIRT2..SIRT7	Sirtuins 1-7
SREBPs	Sterol-regulatory element-binding proteins
ssDNA	single stranded DNA
STAT3	signal transducer and activator of transcription 3
STEMI	ST-elevation myocardial infarction

TCA	tricarboxylic acid cycle
TERC	template RNA component
TERT	telomerase reverse transcriptase
TGFβ	transforming growth factor beta
TIN2	TRF1 interacting nuclear factor 2
Tis	tumour in situ
TL	telomere length
TMA	tissue microarray
TNFα	tumour necrosis factor alpha
TNM	tumour/node/metastasis cancer staging
TPP1	TINT1, PTOP, PIP1 — POT1-TIN2 organizing protein
TRF1	telomeric repeat binding factor 1
TRF2	telomeric repeat binding factor 2
TRFL	Terminal restriction fragment length
TRPM2	transient receptor potential melastatin-related channel
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UC	ulcerative colitis
UCP2	uncoupling protein 2
UTR	untranslated region
UV	ultraviolet (radiation)
VEFG	vascular endothelial growth factor
VNTR	variable nucleotide tandem repeat
VSMC	vascular smooth muscle cell

1 INTRODUCTION

1.1 Ageing and longevity

1.1.1 Ageing: Increased chance of death as time passes.

Ageing is familiar to everyone and easy to describe in an individual but surprisingly difficult to define in the generality, and particularly to define in terms of its underlying causes and mechanisms, even by experts in the field(Hayflick 2007). With increasing numbers of older people in the population worldwide, ageing is an area of growing interest for policy makers at international and all other levels of organisation(United Nations Department of Economic and Social Affairs 2008; WHO 2011). It is a constant consideration for clinicians treating a patient population whose average age is increasing as the lifespan, and the healthspan, or healthy lifespan, of the general population increases(House of Lords Select Committee 2013). Ageing is a fascinating conundrum with ramifications in many scientific disciplines, and notably and more commonly, across boundaries between disciplines. Ageing encompasses a cumulative deterioration in function and resilience in many systems as well as effects, which are obvious, but of no clear functional significance such as skin wrinkling and loss of hair or hair colour. Despite significant investment over the past 25 years, no reliable single or composite measure has conclusively replaced chronological age in assessing functional or biological age of the organism.

Ageing is a cumulative, stochastic, deleterious sequence of random events. Living organisms differ from inanimate objects in the capture of energy and conversion of this energy to a form that is used in a purposeful manner to maintain the integrity of the organism and for growth. However, the organism is constantly exposed to the effects of the non-self environment, which tends to break down its integrity either to use it as a source of energy (predation, infection) or in a random fashion (injury, ‘wear and tear’ e.g. contact with moving particles, or incident electromagnetic radiation). There is a degree of uncertainty always inherent in the processes of molecular level interaction, even though the organism may have evolved to develop a degree of control at this same level. The build-up of the deleterious effects of external forces constitutes ageing. The consequences of ageing are ubiquitous, incident on all systems,

including and pertinently, those responsible for repair and maintenance of cellular machinery.

Longevity, on the other hand, is determined by the robustness to withstand these events. This is in large part a feature of the mechanisms that repair damage to cellular machinery on a molecular level, but there are other elements involved. Longevity is subject to evolutionary selection like any trait of a living organism. Selection pressures integral to the evolutionary process are already well known and centre around the efficacious immortalisation of the organism's genetic code, in its ability to reproduce successfully. Relevant attributes of the organism include its ability to use available energy sources efficiently, to balance the availability of energy with energy-expensive periods in its lifecycle, to minimise damage to its component parts through avoidance of exposure or internally by good control of reactive chemicals, among other traits.

Over the past century, several theories have attempted to explain the phenomenon of ageing and variable lifespan between species and within members of the same species. In the literature, these are generally referred to as Theories of Ageing, although it may be more logical to think of them, as Hayflick suggests, as theories of longevity (Hayflick 2007).

Recent reviews of the prevalent evolutionary theories of ageing have pointed out that although they are not accepted by all gerontologists and do not provide a complete explanation for the phenomenon of ageing, they do go some way towards this and provide an acceptable platform for research(Le Bourg 2001; Ljubuncic and Reznick 2009). The main Theories of Ageing are discussed below.

1.1.2 Theories of ageing

1.1.2.1 Accumulation of mutations. Stochastic: conservation of energy is never 100% efficient.

As time goes on mutations accumulate despite the organism's best efforts at repair. Ageing as described in this way is thus an inevitable process. Following from this is the inference that ageing is a process outwith genetic control, and not a characteristic that can be selected for(Medawar 1952). Likewise there is no evolutionary means to select out organisms which exhibit traits that cause

declining function in old age as they will have passed on these traits by the time they become apparent and evolutionarily relevant (i.e. by the time they have an adverse effect on mortality). Thus traits which allow/promote ageing (decline in function over time) will accumulate in organisms of a species over time, unless these traits also have other effects which expose the organism to other evolutionary selection pressures.

1.1.2.2 Pleiotropic antagonism

The recognition that genes may have more than one effect on survival gave rise to the theory of antagonistic pleiotropy (Williams 1957). This describes the evolutionary selection for traits, which, while they confer a survival advantage at younger ages, have deleterious effects with the passage of time. These may involve, for example, an evolutionarily beneficial acceptance of damage in one system, which increases the chances of the whole individual reaching sexual maturity. The difference between this theory and that of mutation accumulation is that these traits are selected for because of their beneficial effects in earlier life.

1.1.2.3 Disposable soma

Given finite energy resources, the more investment is made in maintenance of the soma, the less remains for reproduction. Obviously a certain amount of maintenance of both components is necessary for the organism to reproduce successfully, but the balance may be subject to modification in different individuals and this balance may be subject to evolutionary selection(Kirkwood and Rose 1991). Thus individuals that strike the optimum balance will be evolutionarily more successful, with more progeny and a lifespan tailored to the duration of reproductive (and childrearing) capability.

1.1.2.4 Rate of living, free radical theory

It has been noted for a century that animals with higher metabolic rates often have shorter life spans. The “rate of living” hypothesis proposed that organisms with higher basal metabolic rates had a shorter lifespan. The mechanistic explanation for this observation was initially unclear. It was speculated that free radicals produced in the cell by metabolic reactions caused damage and this

accumulated over time(Harman 1956). This initiated a fruitful line of research. The discovery of superoxide dismutase as the first enzyme evolved to dispose of free radicals gave support to this theory. Further research has shown that because of their intrinsic reactivity, free radicals are very useful intracellular signalling molecules. However, also because of their reactivity and the danger they pose to cell components, there is a certain amount of damage caused by their use.

As the rate of reactions within a cell increases, the damage produced by molecular activity will also increase. This may not happen proportionately, as cells may develop mechanisms to limit the risk of using reactive oxygen species (ROS), but it seems logical that if the rate of reactions increases to an extreme, eventually the rate of damage will begin to increase also. Reactive chemical species will have the greatest effect, but increase in the rate of any reactions will increase ‘wear and tear’ on that system. Reactive nitrogen species are garnering interest as they appear to pose similar risks to ROS(Afanas'ev 2010).

This theory may partly explain the protective effect of caloric restriction, and the toxic sequelae of nutrient excess. Nutrients not needed for immediate use must be converted to storage forms, with concomitant wear and tear on molecular machinery. Storage forms of nutrients are less reactive; there may be unwanted effects of the (more reactive) nutrient substances awaiting conversion. This will be discussed later in the context of “inflamm-ageing”, the concept of caloric restriction and the inflammatory effect of obesity.

1.2 Cancer

1.2.1 Ageing and cancer

The incidence of cancer varies with age but it is much commoner in older people. Ageing increases the susceptibility to cancer. Cancer is thus an age-associated disease; this may not be the same as a ‘disease of ageing’.

Childhood cancers are a very heterogeneous group and they are much rarer than cancer in adults. They also tend to originate from different groups of tissues, which are often postmitotic (neural, bony) or of foetal origin. Childhood cancers

may be caused by single gene defects, and gave support to the 'two-hit' hypothesis, whereby an inherited predisposition to cancer may be explained by inheritance of a mutated form of a 'tumour suppressor gene'. Tumour suppressor genes are of vital importance in cell cycle regulation and cell death, and in many cases have helped elucidate cell cycle processes. Mutation of the remaining normal allele can occur at a young age and results in the development of cancer.

The classic example is the retinoblastoma gene, named for the cancer which most commonly results from its mutation(Murphree and Benedict 1984). Inherited retinoblastoma accounts for one third of cases and most cases of bilateral disease. The retina is the most commonly affected tissue as it is subject to high levels of incident UV irradiation. Retinoblastoma survivors are prone to higher levels of other forms of cancer in adulthood as well. The retinoblastoma gene is located on chromosome 13. It binds transcription factors of the E2F family, holding the cell in G1 phase. It prevents progression to S phase by inhibition of E2F-related transcription but also because the complex with E2F attracts histone deacetylases to chromatin, further reducing gene transcription. The retinoblastoma protein is thus involved in the DNA repair response and in apoptosis.

In adult cancers, the commonest tissues of origin are epithelial or haematopoietic, tissues characterised by high rates of cell turnover. Cancer in the old is unlike most other age-associated diseases. It is characterized by certain gains of function at cellular level, rather than the deterioration of function central to other conditions. However, in cancer this is a paradoxical gain of function, caused by more hidden deterioration at the higher levels of strategic regulation of cellular function and proliferation.

Cancer occurs when necessary criteria are met. Ageing makes it more likely these criteria will be met, with associated attenuation of the integration and effectiveness of control systems and homeostatic mechanisms.

1.2.2 Hallmarks of cancer

The development of cancer involves accumulation of damage, dysregulation and some gain of function. The necessary criteria for malignant transformation of a cell were initially described as the Hallmarks of Cancer and more recently updated with increased understanding of changes at the molecular level(Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

1.2.2.1 Sustaining proliferative signalling

Normal cells exist in an environment replete with chemical signals, a proportion of which support growth and are essential for the normal development of each cell. Anoikis is the process of cell death instigated in the absence of extracellular membrane attachment, an essential process in the maintenance of cellular proportions for tissue homeostasis. Cells in culture fail to propagate in the absence of suitable signals, and of a suitable growth stratum. Cancer cells are notoriously resistant to anoikis. Cancer cells also exhibit striking loss of the normal dependence on external growth signals. In some cases, they synthesise the growth factors they require, in other cases, mutated transmembrane receptors for such factors are constitutively 'on' and in some cases, the intracellular signalling cascades that translate the signals into molecular activity are altered. The SOS-RAS-RAF-MAP kinase pathway has been the focus of much research in colorectal cancer, as a high proportion of cancer cells exhibit mutations of the Ras protein. Cancer cells may also affect the paracrine signalling of stromal cells in such a manner as, for instance, to supply necessary growth signals.

1.2.2.2 Evading growth suppressors

Growth inhibition is the converse balancing control to cell proliferation which adds accuracy and flexibility. Diffusible molecules, transmembrane receptors and signalling cascades are also involved and can be subverted to allow independence of a different kind from these external signals. Specifically however, cells usually respond to growth inhibitory signals by entry into G_0 (quiescent phase) from which they can return to G_1 under the correct circumstances, or by entry into a post-mitotic state where they relinquish future proliferative capacity. Almost all response to antiproliferative signalling involves the retinoblastoma (Rb) protein pathway, hence its central importance in cell

cycle control and the widespread effects of its mutation and inactivity. TGFβ reduces phosphorylation of Rb protein, increasing the proportion of the underphosphorylated form which is instrumental in maintaining cells in G1 growth arrest(Laiho, DeCaprio et al. 1990) and its importance in colorectal cancer will be discussed in later sections of this chapter. c-Myc promotes entry into a terminally differentiated postmitotic state and is also of significant interest in chemotherapeutic strategies for many cancers.

1.2.2.3 Resisting cell death

Apoptosis is the classic method of programmed cell death, of central importance in embryologic development but retained throughout adult life as a means of disposing of damaged or unnecessary cells without initiation of an inflammatory response. A specific sequence of events breaks down cell membrane, chromosomes, nuclear membrane and nuclear and cytosolic skeletons over 30-120 minutes and the cell components are phagocytosed over the following 24 hours by neighbouring cells. Apoptosis requires sensors, to detect appropriate triggers, and effectors, to initiate and control the dismantling of the cell. Sensors (ligand/receptor pairs) promoting survival include the insulin-like growth factor types 1 and 2 (IGF-1/2) and their receptor, and those promoting cell death include FAS and TNFα and their receptors. Effectors include caspases and p53. p53 is very commonly mutated in cancers, with loss of its proapopototic effect leaving cells more vulnerable to unregulated proliferation.

1.2.2.4 Enabling replicative immortality

Normal cells demonstrate limited replicative potential and after a relatively conserved number of population doublings, cells in culture enter a non-proliferative state termed replicative senescence(Hayflick 1965). Inactivation of p53 and Rb allow further cycles of cell division but cells soon enter a period of ‘crisis’ characterised by massive cell death and end-to-end chromosomal fusions. A tiny proportion of cells emerge from this process with the ability to continue to proliferate and are said to be ‘immortalised’. Cancer cells reset the ‘mitotic clock’ that measures population doublings but it is not clear how they circumvent the onset of senescence in becoming immortalised. The state of

senescence is has been induced in certain groups of cells in conditions of stress or by overexpression of certain genes, as well as by repeated cell division.

1.2.2.5 Inducing angiogenesis

Cells depend for the delivery of oxygen and nutrients on the capillary vasculature and proliferation therefore requires a concomitant expansion in the vascular supply. Angiogenesis appears in many tumours at an early-to-middle stage, before an explosive growth period and is activated by means of an 'angiogenic switch' which alters the balance of pro- and antiangiogenic factors in the microenvironment to promote and sustain growth of new capillaries. Different tumours achieve this in different ways but all with the fundamental aim of supporting the metabolic demands of neoplastic tissue.

1.2.2.6 Activating invasion and metastasis

Tissue invasion and subsequent metastasis is the pathological hallmark of distinction between a benign and malignant lesion. It is dependent on the earlier hallmarks. It indicates an altered relationship with neighbouring cells and with the remainder of the organism (which may by this time be considered a separate 'host'). At a microscopic level, physical coupling with the extracellular matrix and the adjacent cells is altered, and expression of extracellular proteases is required. Interactions with adjacent cells and with the extracellular matrix have regulatory effects that must be ignored by cancer cells. Protease secretion is usually tightly controlled to maintain integrity of the microenvironment and this is one of the most significant changes in cancer cells.

1.2.2.7 Emerging hallmarks of cancer

Inflammation was proposed as the seventh hallmark of cancer (Colotta, Allavena et al. 2009). It is not strictly speaking a characteristic of cancer cells but was accepted by as an important 'enabling characteristic'(Hanahan and Weinberg 2011). Inflammation causes the release of proangiogenic signals and extracellular-matrix-modifying enzymes which promote angiogenesis and invasion. Inflammation alters the balance of prevailing growth factors (growth inhibition signals are reduced and growth promotion factors emphasised) so the

microenvironment becomes more supportive of neoplastic cells. Release of ROS with mutagenic effect can also accelerate development of cancer cells.

Genetic instability and metabolic reprogramming are two important emerging characteristics of cancer cells which, with inflammation, are further discussed in later sections.

1.2.3 Colorectal Cancer

Colorectal cancer is common. With an incidence of 40,700 in the UK in 2010 it is the third commonest cancer in both males and females. It is commoner in older people; 80% of cases occur in people aged over 60 years. Although survival has improved over the past 5 years, five year survival varies from 93% if the cancer is Dukes' Stage A at diagnosis and 6% if Dukes' Stage D. According to latest figures available (2010) 9% of cases are diagnosed at Dukes' A and 9% at Dukes' D. 34% of cases have unknown stage at diagnosis. As colorectal cancer screening programs have been introduced, an increasing proportion of cases are diagnosed at earlier stages.

1.2.3.1 Familial colorectal cancer

5% of colorectal cancer cases are associated with recognized genetic defects, where the 'first hit' is already present in the form of a germline or acquired mutation in one allele of the relevant gene. 1% of cases are due to familial adenomatous polyposis (FAP). A mutation in the APC (adenomatous polyposis coli) gene which codes for the APC protein, controller of β-catenin, is present in FAP and attenuated FAP. In attenuated FAP, the less severe form, the APC protein has impaired function so the effects are less florid. In autosomal recessive FAP, the mutation is in a separate protein, MYH glycosylase, which is involved in base excision repair.

Hereditary non-polyposis colorectal cancer (HNPCC) was reported by Lynch in 1966 and is caused by an autosomal dominant defect in DNA mismatch repair genes. There is also a higher risk of other cancers (uterus, small intestine, ureter and renal pelvis). Currently the Amsterdam II criteria are used to define non-FAP familial colorectal cancer. 35% of families fulfilling the Amsterdam criteria do

not have a demonstrable DNA mismatch deficiency and are sometimes diagnosed with ‘familial colorectal cancer Type X’.

Patients can be stratified according to family history and those identified as high- or –moderate-risk may be offered regular colonoscopy investigation according to protocols determined by the British Society of Gastroenterology (BSG) and the Association of Coloproctology for Great Britain and Ireland (ACPGBI) (Cairns, Scholefield et al. 2010).

1.2.3.2 Non-familial colorectal cancer

Most cases of colorectal cancer are non-familial and are termed sporadic. No specific genetic cause for these cases is identified. They are multifactorial and often occur against a background of increasing tissue dysfunction. Considered from a histological rather than a genetic viewpoint, the development of pre-malignant and then malignant change in colorectal epithelium is well-described in the colorectal adenoma-carcinoma sequence (Leslie, Carey et al. 2002). Research continues to identify important pathways involved and to clarify the physiological functions of these pathways and their components, and the pathophysiological effects of dysfunction.

However, the high turnover of intestinal mucosal cells means that the lifetime of differentiated colonic epithelial cells is 4-5 days. They do not live long enough to develop the mutations necessary for malignant transformation.

1.2.3.3 Colonic stem cells

Colonic stem cells are more properly termed multipotent adult stem cells and are the source cells that continuously regenerate the colonic epithelium. They are found at the base of crypts of Lieberkuhn, where their ‘stemness’ is maintained by the niche in which they are located(Zeki, Graham et al. 2011). The spatial and chemical characteristics of the niche are controlled by pericryptal myofibroblasts located below the basal lamina(Powell, Mifflin et al. 1999). This important epithelial-mesenchymal interaction foreshadows the fundamental relationship of colonic epithelial cells with their environment, which becomes dysfunctional in the development of colorectal cancer.

Colonic stem cells are capable of symmetric and asymmetric cell division. Dividing asymmetrically they form one daughter stem cell and one daughter cell destined for terminal differentiation. They can also divide symmetrically to form two daughter cells destined for differentiation, although this must happen relatively rarely as it would reduce the stem cell population. Symmetrical division to produce two daughter stem cells is also possible and is likely to be important in the repair and resolution of cytocidal damage of any sort affecting the colonic epithelium.

A small population of colonic stem cells exists at the base of each crypt. As these divide, their descendants move up the crypt, differentiating into one of the four cell types within the colonic epithelial population. Cell division does not occur at an equivalent rate among all stem cells, and in fact, varies among them so that at any one time, the crypt is populated by clonal expansion of the progeny of only one stem cell. Differentiation of cells progressing up the crypt is tightly controlled and important regulators are the Wnt/β-catenin family (Kosinski, Li et al. 2007; van der Flier and Clevers 2009). The importance of the APC gene in the development of colorectal cancer is therefore obvious. Members of the bone morphogenetic protein family are thought to exert a growth-inhibitory effect and are expressed at higher levels towards the top of crypts(Kosinski, Li et al. 2007; Hardwick, Kodach et al. 2008).

Obviously, if the one stem cell dividing to replenish the differentiated cell population develops a significant degree of damage, there is propagation of a clone of cells carrying important mutations with a clear risk of development of cancer. Stem cells already show some of the necessary hallmarks of transformed cells. Functions necessary for epithelial repair mean that several more of the hallmarks are within their repertoire or the repertoire of the cells which support the stem cell niche.

If the resident population of stem cells is reduced significantly, replacements will be recruited from the haematopoietic stem cell compartment. TGFβ is central to this process(Sipos, Valcz et al. 2012).

1.2.3.4 Cancer stem cells

This term can be used in different ways. It can be used to indicate stem cells which have become transformed. Alternatively, the concept of a stem cell as one which maintains a cohort of cells can be applied to the cancer as well as to the colonic epithelium. In this sense cancer stem cells are a subdivision within the tumour cell population which are able to survive many forms of treatment and divide to produce new populations of tumour cells, not all of which may have the full complement of functional capabilities of the stem cell parent. Treatments may in effect select for these cells and by killing less virulent cells, provide them with a selective advantage.

1.2.3.5 Ageing in colonic mucosa

Although the effect of age on colonic mucosa is not completely understood, several aspects have been described(Sipos, Leiszter et al. 2011). Colonic stem cells may be reduced in number over time and their response to injury was less effective in a mouse model(Kirkwood 2004). Altered methylation patterns of several genes with age indicate that there is an epigenetic effect of ageing. Oestrogen receptor 1 (ER1) shows age-dependent methylation in colonic mucosa; it may predispose to tumour formation by dysregulation of colonic epithelial cell growth and differentiation. Methylation of ER1 is more common in ulcerative colitis patients with colonic carcinoma than those without(Tominaga, Fujii et al. 2005).

Response to growth factors is also altered in older colonic epithelium. Age-related increase in epidermal growth factor receptor is associated with increased colonic cell proliferation in rats(Malecka-Panas, Relan et al. 1996). Hepatocyte-derived growth factor is produced by mesenchymal cells and acts on epithelial cells expressing the appropriate receptor. It is important in gut epithelial cell homeostasis and gastrointestinal wound healing(Sipos, Galamb et al. 2008). In vitro expression by fibroblasts increases after 70% of lifespan in culture.

Telomere length has been shown to be shorter in chronologically aged colonocytes. Telomerase is normally expressed in stem cells in the base of colonic crypts but in some cases telomerase activity is reduced in conjunction with ulcerative colitis(Usselmann, Newbold et al. 2001; Kleideiter, Friedrich et

al. 2003). Telomere length as a marker of biological age in colonic epithelium and other tissues will be discussed in more detail in later chapters.

1.3 Cancer and Inflammation

Cancer risk in several tissues is noted to be increased in the context of chronic inflammation. The link between inflammatory bowel disease of extensive anatomic involvement and prolonged chronologic duration is well documented(Lashner, Silverstein et al. 1989; Eaden, Abrams et al. 2001). Barrett's oesophagus is a condition of lower oesophageal metaplasia thought to develop in response to chronic acid-induced oesophagitis which is strongly associated with oesophageal adenocarcinoma. Chronic inflammation resulting from smoking probably contributes to the development of lung adenocarcinoma, as well as the effects of carcinogens in smoke. In histological assessment of urothelial lesions, it is recognised that it may be impossible to conclusively differentiate reactive from dysplastic lesions and a separate category of Urothelial Atypia of Unknown Significance allows for such cases to be identified for closer followup(Reuter, Epstein et al. 1999).

Repeated episodes, or chronic continuous inflammation results in repeated tissue damage. There is an ongoing requirement for repair and cell division to replace damaged tissue and the cellular microenvironment is permissive for repeated cell division. Increased rates of cell division will increase the chance of cancer cells escaping the stringent controls on propagation of damaged cells.

The body has numerous mechanisms to identify and manage cells under stress, or which have sustained damage. Most of these operate in the context of inflammation, which is the most ancient defence system in the evolutionary armamentarium.

1.3.1 Inflammation: A brief overview

Inflammation is the most basic form of defence seen in multicellular organisms. It has been developing for 800 million years and is both a complex and well-integrated process. The inflammatory response works closely with the innate immune system in the defence of the organism.

The initial insult triggers a local inflammatory response, which may be dealt with by tissue-resident cells. If this is not possible, other cells will be recruited and the response may become systemic.

Generically, the inflammatory process involves induction, effect and resolution. The aim is to nullify the external threat, and return to baseline homeostasis. The initial trigger for the inflammatory response can be categorised as infective, or resulting from tissue death, or from tissue stress and dysfunction. The sequelae of this last trigger are referred to as para-inflammation, indicating the intermediate nature of the damage.

The inflammatory response involves several interlinked pathways with many component parts; it has cellular and humoral arms. As such, it can be useful to classify inducers, sensors, mediators and effectors of inflammation(Medzhitov 2008). Different triggers of inflammation invoke a variable subset of downstream responses, tailored (to a certain degree) to the initial insult. The resolution phase also requires a similar set of response elements.

Inducers of the inflammatory response can be exogenous or endogenous. Exogenous inducers are pathogen-associated molecular patterns (PAMPs) or virulence factors. Virulence factors are not recognised by chemical interaction, but by their effects, such as the potassium-efflux resulting from pore-forming exotoxins of Gram-positive bacteria. PAMPs are organic particles of pathogen origin which are recognised by evolutionarily conserved pattern recognition molecules (PRMs).

Endogenous inducers of the inflammatory response are less well-defined but desequestration has a potent effect. Desequestration refers to contact between substances normally kept separate in the functioning cell, such as ATP, K+ ions and members of the calcium-binding protein families. Epithelial and mesenchymal cells are normally separated from each other by a basement membrane and ‘unscheduled contact’ can also be a powerful trigger for an inflammatory response. Epithelial and mesenchymal cell interaction is also central in determining colonic stem cell differentiation as discussed earlier.

Inflammation has a positive protective effect overall, but the actions of effector cells and molecules are not specific and there is an inherent degree of damage to surrounding cells on evocation of the inflammatory response. Repeated or sustained inflammation may overcome the ability of the organism to completely repair the damage due to the triggering insults or to the inflammatory response itself. This is one important source of ageing and of increasingly damaged repair systems.

The inflammatory response is linked to epithelial cell function on a constitutive level as well. Tissue-resident macrophages make up approximately 10-15% of the cell population and are intrinsically involved in maintaining the tissue microenvironment. They constantly monitor cell state and can detect variation in cell stress by expression of specific peptides on the cell membrane.

Cell state is generally categorised into 4 levels of increasing stress; transit between these levels is by specific genetic ‘switches’. The stages are basal (normal and unstressed), stressed, apoptotic and necrotic. Although necrosis was thought to be an unregulated event, recent evidence appears to suggest that there may be some underlying regulated ‘emergency plan’. Switching between these states as a distinct action ensures that the cell does not give out mixed messages and also that it exhausts all possibilities of remediation before switching to the next, less desirable state.

The significance of macrophage monitoring of cell state is twofold. Macrophages have a certain capacity to support cells by release of growth factors and modulation of the microenvironment and may be able to prevent deterioration and change of state by doing so. Equally, if a cell is reverting to apoptosis or necrosis, macrophages are central in coordinating the appropriate response, with or without the induction of an inflammatory response. Macrophages, with their regulatory effect on epithelial cell growth and maintenance, and also on angiogenesis and extracellular matrix breakdown can also be co-opted in tumour development and metastasis. The important role that macrophages play in this situation has been reviewed (Condeelis and Pollard 2006) and is the subject of more recent study. Effects on survival in cancer are varied, and probably reflect the variable functions of macrophages in the tumour microenvironment (Mohammed, Going et al. 2012; Richards, Flegg et al. 2012).

1.3.1.1 Local inflammatory response

The initial inflammatory response will be local. Ideally the external threat is nullified at this stage and localised damage is repaired by the organism. Recovery requires cell division to replace cells lost or damaged. If the threat overcomes local resources, humoral signals stimulate an escalated response which may become systemic.

1.3.1.2 Systemic inflammatory response

A truly systemic inflammatory response induces measureable changes in a wide range of serum markers. Hepatic synthesis of positive 'acute phase proteins' (APPs) increases and there is a reduction in serum levels of negative APPs due to increased catabolism and decreased synthesis. The systemic inflammatory response induced by infection is more completely understood than the chronic systemic inflammatory response to other pathological processes. The systemic response to cancer may reflect a modulation of a chronic inflammatory response already present and instigated by an inflammatory pathology predisposing to the cancer or it may be almost solely due to the cancer itself.

NFκB appears to have a central role in the systemic response, whatever the causal insult.

1.3.1.3 Inflamm-ageing: inherent systemic inflammatory response?

Inflamm-ageing refers to the age-associated changes in the innate immune system, which can be summarised as a progressive loss of regulatory control of the adaptive immune system and general upregulation of the innate immune system with time(Cevenini, Caruso et al. 2010). As many systems show progressive low-level dysfunction there are correspondingly more endogenous sources of inflammation in the aged organism. There is also a less appropriate inflammatory response from the similarly affected (innate) immune system.

1.4 Fates of aged - damaged - cells

1.4.1 Apoptosis

Programmed cell death is a planned and carefully orchestrated process resulting in the removal of a cell. It is seen in embryological life as body parts take on their final form. For instance, it occurs in the digital web spaces to create the digits and is thought to explain the recanalisation of the gut. There is a minimum of inflammatory reaction and there are characteristic changes in the nuclei and cytoplasm preceding the breakdown of the cell and nuclear membrane allowing phagocytosis of cell components for recycling. If control of apoptosis is lost, cells may proliferate or survive despite having sustained significant damage.

1.4.2 Anoikis

Anoikis is programmed cell death in response to loss of contact with the extracellular matrix. Organisms have evolved with the threat of cancer over the course of generations and anoikis is a fundamental defence against cancer. It means that not only must the malignant cell escape from external controlling signals, but simple lack of those signals itself invokes cell death, and this process too must be overcome.

1.4.3 Necrosis

Pathological cell death results in cell lysis and release of contents stimulating an inflammatory reaction. It is associated with the characteristic macroscopic features of erythema, swelling, pain and heat. At a cellular level there is vasodilation, increased capillary permeability and oedema. Inflammatory cells are recruited and debris cleared by phagocytosis as part of the reaction.

1.4.4 Transformation

A very few aged and damaged cells achieve transformation to become immortalised cancer cells. Characteristics of these cells were described earlier. The path of their development is an important focus of cancer research: identification of essential and decisive changes at a cellular level will guide the development of novel effective and precise therapeutic strategies with minimal damage to surrounding cells. This is likely to be increasingly important to preserve remaining function in more aged patients.

1.4.5 Senescence

1.4.5.1 The Hayflick Limit

Leonard Hayflick (Hayflick and Moorhead 1961) described a finite and similar number of cell divisions undergone by repeatedly examined cultures of human cells. This phenomenon became known as the Hayflick Limit. It was predicted by Olovnikov as a result of the end replication problem due to the mechanism of DNA replication(Olovnikov 1973). The Hayflick Limit was explained by the discovery of telomeres (Blackburn and Gall 1978); it was suggested that they acted as a replicometer or 'mitotic clock', which induced cell cycle arrest after a prescribed number of cell divisions. However telomeres are much more than a length of expendable DNA, as will be discussed in later sections.

1.4.5.2 Organ senescence

Increasing numbers of senescent cells affect organ function by mass effect through reduction in the number of normally functioning cells. 'Senescence' as applied to organs describes progressively reduced function over time.

1.4.5.3 Cellular senescence

Senescent cells enter a state of irreversible growth arrest. There are many triggers for this process but critically short telomeres are one of the most powerful. DNA damage is also a potent inducer of senescence(von Zglinicki, Saretzki et al. 2005). Shortened telomeres are associated with significant genetic anomalies such as chromosomal translocation or fusion. The phenomenon of senescence-induced growth arrest is thought to constitute a defence against cancer; thus cells with significant DNA damage, or critically shortened telomeres are prevented from passing on their damaged DNA to daughter cells.

1.4.5.4 Senescence-associated secretory phenotype

As research into the phenomenon of senescence accumulates, it has become clear that senescent cells, while no longer dividing, are not merely passive. The damage that has accrued can cause them to continue to function within their niche but this function may be aberrant, with the potential accumulation of abnormal proteins or other molecules. A characteristic senescence-associated secretory phenotype is recognisable and this appears to have a pro-inflammatory

paracrine effect(Campisi 2013). Thus, senescent cells within a tissue may alter the microenvironment. The effects of their paracrine secretions (while not yet clear) could support or promote malignant change in neighbouring cells.

1.5 Markers and Mediators of Inflammation

1.5.1 CRP

CRP is a short pentraxin, member of one of the fluid-phase families of pattern recognition molecules which recognise PAMPs. CRP is the major acute-phase reactant in humans(Mantovani, Valentino et al. 2013). Neutrophils store CRP for release as an immediate response to injury. It is also synthesised by macrophages in response to gene-activation, and by hepatocytes in a slightly delayed systemic response, on stimulation by IL-6. Levels are usually <3mg/ml in normal human serum but can increase a hundredfold within 6 hours in response to insult.

1.5.2 Albumin

Albumin is synthesised by the liver and is the most abundant plasma protein. It has important roles in acid-base balance, in binding and transport of drugs, hormones, ions (including calcium, iron and copper), and metabolites including amino acids and fatty acids. Albumin is an important part of extracellular defences against ROS-induced stress, due to its volume and a biochemical structure which allows it to bind copper, iron and hydrogen ions and free radicals, undergoing oxidation of a thiol group in the process(Sitar, Aydin et al. 2013). Albumin also has an inhibitory effect on platelet aggregation.

It is a negative acute phase protein, with synthesis reduced as part of the 'hepatic reprioritisation' response to proinflammatory cytokines, and deficiency of specific amino acids. Albumin levels do not respond to nutritional supplementation immediately but rather to the resolution of the cause of inflammation(Hulshoff, Schricker et al. 2013). Albumin replacement is used in critically ill patients but it may be that improved outcomes are due to other actions apart from its ability to maintain colloid osmotic pressure, and it is not clear that all patients experience benefit from its use over crystalloid for fluid resuscitation(Cochrane 1998).

Despite its poor reflection of nutritional status, and because this is due to the numerous other competing effects on serum level, particularly relating to the systemic inflammatory response, hypoalbuminaemia has for some time been recognised as a risk factor and predictor of general debilitation and poor prognosis in patients with sepsis and cancer as well as after surgery (Herrmann, Safran et al. 1992; Daley, Khuri et al. 1997; Don and Kaysen 2004).

1.6 Colorectal Cancer and inflammation

1.6.1 Inflammation as a defence in the gut

The gastrointestinal epithelium provides a single-cell barrier between the controlled internal milieu of the organism and the external environment, and harbours a rich commensal microbiological flora as well as a multitude of pathogenic organisms(Elinav, Henao-Mejia et al. 2013). The large surface area of this barrier provides for essential physiological functions but also offers a significant risk of breach with potential life-threatening systemic infection. Inflammation is a fundamental second-line of defence, and constant low-level activation is probably necessary to maintain a healthy micro-environment within the epithelium and submucosa. Likewise, inflammatory responses may have some success in recognising and eliminating dysplastic cells in the earliest stages of their development(Rizzo, Pallone et al. 2011).

1.6.2 Increased cancer incidence in chronic inflammation: IBD and cancer

An increased risk of colorectal cancer in association with inflammatory bowel disease has been noted for some years(Lashner, Silverstein et al. 1989; Lennard-Jones, Melville et al. 1990; Eaden, Abrams et al. 2001). Mechanisms which may underlie this include inflammation induced by commensal species as well as pathogens, inflammatory oxidative stress, p53 mutation, telomere shortening and genetic instability, with alteration in the relationship between mucosal and submucosal cell populations(Okayasu 2012). In ulcerative colitis, there is a higher risk of CRC in pancolonic inflammation after 15 years, which justifies increased colonoscopic screening and subtotal colectomy in some patients(Connelly and Koltun 2013). Cancer has been observed in chronically inflamed tissue associated with Crohn's disease as well (Zagoni, Peter et al.

2006), although the discontiguous nature of the disease process has made it more difficult to quantify the extent of inflammation and draw reliable associations between the extent of inflammation and neoplastic change.

1.6.3 Dysregulation of inflammation in the gut

Ageing is the most potent risk factor for colorectal cancer as it 'represents the general framework within which the tumour environment evolves'(Garagnani, Pirazzini et al. 2013). Inflamm-ageing may increase the likelihood of a dysregulated response to the recurrent threats posed to gastrointestinal epithelial integrity. Chronicity of inflammation may in itself be a risk factor for dysregulation as there are more opportunities over time for control to be impaired.

A direct link between inflammation and colorectal epithelial cell proliferation has been observed in a mouse model deficient in components of the inflammatory response, with colitis-associated colorectal cancer. The NLRC4 protein activates an inflammatory response to Gram-negative bacteria common in the gut. Unexpectedly increased tumorigenesis in NLRC4-deficient mice was not due to alteration of inflammatory pathways but to enhanced proliferation and attenuated apoptosis(Hu, Elinav et al. 2011). It is not clear what the underlying mechanism for this interaction is, but further analysis of the process will likely be informative.

1.6.3.1 Anti-inflammatories and CRC

Given the evidence on the involvement of inflammation in CRC, the role of non-steroidal anti-inflammatory agents (NSAIDs), has also received extensive scrutiny. A National Institute for Health Research Health Technology Assessment review of aspirin use for primary prevention of CRC confirmed a small effect in reducing incidence, evident after 5 years of use(Sutcliffe, Connock et al. 2013). Initial studies were generally performed in patients on treatment with aspirin for cardiovascular disease and aspirin use for primary prevention has been limited by incidence of gastrointestinal bleeding.

Aspirin is a non-selective cyclo-oxygenase (COX) inhibitor, which at low doses inhibits platelet function by irreversible inactivation of COX-1. It also has

inhibitory effects on NFκB and Wnt/β-catenin signalling. Its effectiveness against colorectal cancer at doses which affect platelet function suggests that colorectal cancer and atherothrombotic disease may share pathophysiological processes related to platelet activation(Dovizio, Bruno et al. 2013). This is an interesting concept, particularly in view of relationships between the metabolic syndrome and ageing, and between fetuin A (one of the subjects of this thesis) and cardiovascular disease. These interactions are discussed in more detail later.

Cyclo-oxygenase 2 (COX-2) is an early response gene inducible by a range of inflammatory stimuli, encoding an enzyme which catalyses a rate-limiting step in prostaglandin synthesis. Levels of COX-2 are elevated in a large proportion of colorectal tumours. Selective COX-2 inhibitors have also been shown to reduce incidence of CRC and resolution of adenomas, particularly in FAP patients. Cardiovascular events have limited treatment to an extent, but studies indicate that the mechanism of action, although unclear, involves global reduction in prostaglandin synthesis, particularly PGE2 (Wang and Dubois 2010).

1.6.4 Inflammatory response to colorectal cancer

Inflammatory processes also have a role to play in the host response to colorectal cancer. At a local level, as indicated earlier, these probably involve recognition and destruction of neoplastic cells detected as abnormal, and may often be successful, in that only the failures continue to develop into clinically evident tumours.

In established tumours, pathologists have for some time recognised that there is often an inflammatory cell infiltrate within the tumour and that the density of infiltration corresponds to a better outcome. The precise nature of the infiltrate, the cells recruited and type of immune response initiated have a bearing on tumour invasion, and response to treatment(Roxburgh and McMillan 2012).

1.6.5 Worse prognosis with systemic inflammatory response in colorectal cancer

A large body of work has shown the detrimental effect of systemic inflammation as a separate marker of poor prognosis in a range of cancers. There seems to be

an inverse relationship between the strength of the local and systemic inflammatory responses, with weak local and heightened systemic responses constituting poor prognostic factors. Tumour necrosis may be one factor triggering a systemic inflammatory response(Richards, Roxburgh et al. 2012). The balance between these two in the response to colorectal cancer probably reflects the pre-existing inflammatory landscape in which the tumour develops.

1.6.6 Modified Glasgow Prognostic Score (mGPS)

Initial studies of several markers of inflammation identified the combination of CRP and albumin as being of particular predictive efficacy, and defined the threshold level for each which is associated with worse outcome. The original Glasgow Prognostic Score gave equal weighting to albumin and CRP, but was modified as CRP appeared to carry greater predictive value(Leitch, Chakrabarti et al. 2007). The modified score awards 1 point for CRP over 10, and a further point if albumin levels are 35 or below.

The modified Glasgow Prognostic Score has been validated in colorectal cancer in several populations, and in many other cancers (Al Murri, Bartlett et al. 2006; Crumley, McMillan et al. 2006; Glen, Jamieson et al. 2006; McMillan, Crozier et al. 2007; Ishizuka, Nagata et al. 2012; McMillan 2013). The poor prognosis of patients with elevated mGPS appears to result from a less effective host inflammatory response to the cancer, in combination with the effect of malnutrition. The malnutrition observed in cancer, particularly in advanced stages of the disease, may reflect the subversion of normal homeostatic mechanisms to the metabolic demands of malignant cells not responsive to signals indicating a need to curb demand for the survival of the organism as a whole.

1.6.7 Obesity, inflammation and CRC

Obesity is recognised as a chronic inflammatory condition and feedback loops set up by visceral adipose-derived molecules such as leptin, resistin and adiponectin can increase the background proinflammatory state of the gut(Vazzana, Riondino et al. 2012). Macrophages resident in obese adipose tissue contribute by

secretion of proinflammatory cytokines, as distinct from the secretory patterns of macrophages in lean adipose tissue(Yehuda-Shnaidman and Schwartz 2012).

Obesity is only one of several metabolic factors in the aetiology and pathophysiology of cancer.

1.7 Metabolism in colorectal tissue

1.7.1 Normal cellular respiration

Glucose is the carbon source for 80% of phosphorylated compounds in cells so it is difficult to overstate its importance in control of metabolism and secondarily in control of cell proliferation. Other energy sources are used but in the normal cell, these are tightly controlled. Most cells maintain a small amount of glycogen as a glucose store; glucose itself has a significant effect on several enzymatic reactions and is not freely available within the cell for this reason. Glucose conversion to glycogen is controlled by glycogen synthase, which is inhibited by phosphorylation by glycogen synthase kinase (GSK). The isoform GSK3 is inhibited by PI3K/AKT pathway, which is activated by insulin and the insulin–like growth factor (IGF) family. Recent research on GSK3 has shown its activity in the fields of inflammatory response, apoptosis, cell migration and control of cell adhesion.

Glycolysis (fermentation) and aerobic respiration are the two main energy-releasing pathways used by the normally functioning cell. Glycolysis converts glucose to pyruvate which is reduced to lactate and excreted in the bloodstream, with the generation of 2 molecules of ATP per molecule of glucose used. It takes place in the cytosol. Aerobic respiration is a much more complex process which begins with glycolysis in the cytosol and continues by means of the Krebs (or tricarboxylic acid) cycle and oxidative phosphorylation in the mitochondria. Glucose and other substrates are completely oxidised to CO_2 and water, with the generation of 15 times more ATP. There is more likelihood of ROS production but as with all processes that generate ROS, they are generally tightly controlled.

As a rule, fermentation is reserved for short-term energy generation in hypoxic conditions, and with the return of normal oxygenation, aerobic respiration resumes with the oxidation of any lactate that has not been removed by the vascular system. Under these conditions cells, even cardiac myocytes which are specialised to function in intermittent hypoxia, tend to restrict non-essential synthesis and proliferation.

The pentose phosphate pathway (PPP) is the major route for conversion of glucose to 5 carbon sugars which are used in nucleic acid synthesis. Glucose-6-phosphate is converted to ribose-5-phosphate and NADPH, which is essential for fatty acid synthesis, and is a scavenger of ROS.

1.7.2 Metabolism under different conditions of substrate availability

Cells have evolved to cope with a wide variation in the ambient availability of nutrients. Within the parameters of survivable variation in nutrient availability, the redox status of the normal cell will vary in response to external ambient conditions. In multicellular organisms, integrating the function of individual cells with overarching strategies for the survival of the organism is key, but maintaining homeostasis of the extracellular environment to optimise cellular performance is fundamental to achieving this. It is important to consider this difference between single cell model systems and whole organisms in considering metabolic adaptations.

1.7.2.1 Acute fasting

In the acute response to lack of food or nutrients, glucose production is prioritised. There is no mitochondrial biogenesis. Glycogen stores in cells are used and active gluconeogenesis commences in response to glucagon secretion in humans.

1.7.2.2 Prolonged fasting

Prolonged lack of food or nutrients activates additional responses. As hepatic glycogen stores are used up, other energy sources are required to replace them for essential tissues such as brain and skeletal and cardiac muscle. Specific

pathways are activated to suppress hepatic gluconeogenesis. Fatty acid oxidation is activated to obtain the additional required energy substrates initially; amino acid catabolism will also be induced if necessary. There is no stimulus for mitochondrial biogenesis.

1.7.2.3 Caloric restriction

Caloric restriction is defined as a reduction in caloric intake by 30-40% with respect to organisms fed ad libitum, with optimal levels of vitamins and minerals to maintain nutrition. It is thus significantly different from the fasting states described above. It has been noted to produce increase in lifespan in organisms from yeast, nematodes and Drosophila to mice. Caloric restriction can increase the lifespan of laboratory rodents by up to 50%(Sohal and Weindruch 1996). Caloric restriction is not documented in humans; the degree of restriction is difficult to maintain.

Mild and severe caloric restriction have different effects on gene expression profiles. In yeast, caloric restriction triggers a switch from fermentation to the tricarboxylic acid cycle for energy generation. A consideration in extrapolating observations on caloric restriction from unicellular to multicellular organisms is that in a multicellular organism, some cell types will be initially screened from the effects of reduced availability of resources. Essential tissues such as brain, heart, and skeletal muscle will be preferentially supplied with necessary substrates. Thus, there may be tissues which experience severe caloric restriction, at least initially, and tissues which experience milder caloric restriction(Qiu, Brown et al. 2010). Variables of note will include tissue, duration of caloric restriction, state of function of the vascular supply and of overall systemic control systems such as hormonal messengers. In mammals, there is an observed switch from glucose to fatty acid metabolism in caloric restriction.

The mechanism underlying the benefits of CR is still debated. Slowing of development has been discarded as the causal factor as CR can be started at anytime during the lifespan and still confer benefit. It has been postulated that CR works by slowing down metabolic activity and thus reducing free radical damage. Stochastic oxidative damage is increased with increased number of

metabolic processes ongoing in a cell. Active cellular metabolism for any reason therefore incurs more damage and if resources – of energy and raw materials – are insufficient to repair these, then longer-term damage may result.

Caloric restriction reduces the oxidative burden on cells as measured by a reduction in by-products of oxidative damage to fats (lipofuscin) proteins and DNA(Qiu, Brown et al. 2010). However, the metabolic rate does not slow. It may be that there is a gross reduction in metabolic activity, rather than rate. A simplified repertoire of metabolic processes may be required to maintain the steady state in an austere environment with a reduction in free radical damage overall.

Conversely, excess food intake could render glucose or fatty acids, in molecular terms, as much a pollutant as other more familiar toxins. Increased cellular metabolic activity puts more pressure on disposal systems or systems to control reactive oxygen species used as messengers, cofactors, intermediates.

Caloric restriction in SIR2-deleted yeast strains does not produce the increase in lifespan observed in wild-type yeast. SIR2 (Silent Information Regulator Type 2) is a yeast protein evolutionarily conserved across most classes of higher organisms; these orthologues are known as sirtuins. It has been hypothesised that sirtuins extend lifespan in the organisms in which caloric restriction has been observed, by control of metabolic pathways. The involvement of specific sirtuins in metabolic control will be discussed in more detail subsequently (Section 1.15).

Caloric restriction may produce a shift in metabolism in cells allowing them to increase efficiency in energy usage or to delay energy-hungry metabolic processes, such as cell division (Lamming, Wood et al. 2004).

The central effects of caloric restriction focus on the mitochondria: increased mitochondrial biogenesis, and activation of mitochondria to increase respiration rate, increase ATP production and maximise the extraction of energy from chronically depleted resources. This is achieved by use of other fuels such as fatty acids, ketones and amino acids, oxidation of which produces many more molecules of ATP per unit fuel than glycolysis alone and also avoids the

production of acidic products. This activation involves other members of the sirtuin family; in humans the so-called 'mitochondrial sirtuins' are SIRT3, SIRT4 and SIRT5 and are also discussed later.

1.7.3 Control of metabolism: tailoring to prevailing conditions

Strategic control of metabolism is a multilayered hierarchical process, with integration of feedback loops within and between levels. In a comprehensive review (Lindsley and Rutter 2004), 5 pathways are described. These are the AMPK pathway, mTOR, PAS kinase, hexosamine biosynthesis pathway and the networks involving the sirtuins. There is crosstalk between some, if not all, of them. SIRT1 is thought to activate the AMPK pathway indirectly as increasing energy usage depletes stores of ATP and ADP, and it is the change in these molecules that stimulates AMPK activity (Qiu, Brown et al. 2010; Ruderman, Xu et al. 2010). Indeed these molecules are thought to regulate each other. AMPK downregulates processes not imminently required for survival, which consume ATP, while stimulating those that replenish ATP stores, which are generally catabolic in nature.

Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) controls genes involved in fatty acid metabolism. It is inhibited by high levels of Acetyl-CoA after eating and activated (indirectly) by NAD (via SIRT1) on fasting. These two small molecules also modulate chromatin packaging and thus gene expression in opposite directions(Naimi, Arous et al. 2010).

With this degree of complexity, a systems biology approach has been used with success to identify constraining parameters and common scaling laws in cellular metabolism among species(Kriete, Sokhansanj et al. 2006).

1.8 Metabolism and Ageing: Regulation of metabolism and longevity

1.8.1 Dysregulation of metabolism with age

The links between metabolic process and longevity are intuitive since efficient metabolism will enable growth when advantageous, and will maintain essential systems in a good state of repair. Organisms in the prime of life will likewise

exhibit well-regulated metabolic processes capable of timely and smooth adjustment to optimise response to prevailing environmental conditions. Aged and imperfect disposal systems are more likely to allow build-up of toxic metabolites.

The mechanistic descriptions of both metabolic pathways, and control and integration of these pathways are still incompletely understood. Improved understanding of metabolism and control of metabolism with increased agedness is an important health concern in the Western world and is also an increasing problem in countries where populations are becoming more affluent. Excess normal metabolic substrates can also be toxic.

1.8.2 Metabolic syndrome

The 'metabolic syndrome' was first described in 1988, in the Bantling lecture to the American Diabetic Association(Reaven 1988). Reaven described a constellation of co-morbidities and proposed increasing insulin resistance as the unifying underlying factor. The existence and defining criteria of metabolic syndrome are a matter for debate still(Meigs 2000) but include obesity, glucose intolerance, hypertension, high triglycerides and LDL cholesterol, low HDL cholesterol and a proinflammatory and prothrombotic state(Arai, Kojima et al. 2009). Metabolic syndrome and insulin resistance are more common with age(Kobayashi, Nishimura et al. 2007) but centenarians appear to have demonstrable differences in adipokines which modulate insulin sensitivity(Kanaya, Wassel Fyr et al. 2006). Given links between visceral adiposity, insulin resistance and other potential markers of biological ageing (like peripheral blood leukocyte (PBL) telomere length), one theory suggests that the metabolic syndrome may amplify or accelerate the ageing process(Barzilay and Stein 2011).

Of note, there is also evidence to support early programming of metabolic control in response to adverse nutritional states with subsequent increased risk of development of the metabolic syndrome(Gluckman and Hanson 2004; McMillen and Robinson 2005). Certainly, appreciation of a 'big picture' integrating function of brain, liver, gut, pancreas, and adipose tissue will be necessary to understand whole-body energy homeostasis and its effects on longevity. One

such theory integrates hormonal modulation (by oestrogen, androgens and insulin), inflammation and lipid mediators of redox stress and notes epidemiologic evidence in support of the combined effect of these factors on colorectal cancer risk(Slattery and Fitzpatrick 2009).

1.8.3 Mitochondrial dysfunction with age

Oxidative stress has been implicated in the pathophysiology of several components of the metabolic syndrome. Excess production of free radicals or failure to neutralise them efficiently allows damage to accrue. Mitochondria, as the site of much cellular production of free radicals, are in the front line. Damaged mitochondria will also function less efficiently in the handling of free radicals and so a vicious cycle has been described(Frisard and Ravussin 2006). It is logical that different individual susceptibilities and genetic predispositions to mitochondrial disease should underlie the identity of the initiating lesion, but in due course a common positive feedback loop may lead to ongoing damage.

1.9 Metabolism in cancer

Cancer cells and inflammatory cells share challenges incumbent on cells with a high (or unregulated) metabolic rate. However, several specific aspects of cancer cell metabolism have been noted for over 50 years, and renewed interest in these phenomena may produce more complete explanation with the integration of more recent discoveries.

1.9.1 Glycolysis: the Warburg effect.

In 1924 Otto Warburg observed that cancer cells exhibit increased uptake of glucose and extrusion of lactate into the surrounding tissues, with consequent effect on the pH in the vicinity, compared to normal cells(Warburg 1956). This pattern of metabolism is not altered by the presence of oxygen, another difference in comparison to normal cells. His data formed the basis for a lively debate, and for a lifetime of distinguished research on the part of its discoverer and several other scientists. Warburg's research uncovered several facets of cancer cell metabolism, which remain relevant, and indeed are the focus of renewed interest in recent years.

The initial experiments used slices of tumour tissue, whose thickness was thought to have introduced a degree of hypoxia; further experiments also used ascites cancer cells, which live virtually free in the peritoneal cavity and confirmed the observed results in the absence of hypoxia.

More recent studies have also shown that glycolysis is used as the primary mode of energy generation in cancer cells even in presence of other energy sources. Genetic studies have confirmed that enzymes of the glycolytic pathway are upregulated in 76% of colorectal cancer specimens(Yeh, Wang et al. 2008). Administration of glucose increased proliferation in colorectal cancer cell lines and this was also associated with an increase in transcription of eight genes involved in glucose uptake (GLUT-1) and glycolysis.

GLUT-1 encodes glucose-transporter-1; it is consistently upregulated in colorectal cancer cells with K-RAS mutations, which are able to survive in low-glucose conditions(Yun, Rago et al. 2009). These authors found that cells with wild-type KRAS which survived a low-glucose environment tended to express high levels of GLUT-1 and 4% acquired KRAS mutations not present in parent cells.

Why cancer cells should preferentially use a less efficient mode of energy generation is not clear. The data above suggest there might be an advantage in being able to survive in a low-glucose environment such as might arise within a tumour mass without regulated vascular supply to provide adequate nutrients.

Further information comes from a study of HCT116-derived colorectal carcinoma cell lines. These were engineered to express varying levels of a mitochondrial ATP-synthase subunit and showed widespread changes in transcription, protein expression, mitochondrial function and in vivo tumour-forming capability(Sanchez-Arago, Chamorro et al. 2010). Following treatment with oligomycin (an inhibitor of ATP-synthase) cells showed a more aggressive tumour-forming phenotype (termed G cell phenotype). These cells, surprisingly, also showed reduced expression of genes associated with mitochondrial biogenesis and function. Tumourigenicity appeared to be selected for in conjunction with glycolytic tendency and these cells also had a cell-death resistant phenotype. G cells showed increased resistance to 5-FU treatment which was reduced by treatment with an activator of mitochondrial

decarboxylation of pyruvate, whose effect was to reduce aerobic glycolysis to baseline levels.

1.9.2 Specific enzyme involvement in glycolysis in cancer

A recent review of the Warburg effect highlighted several important enzymes(Bayley and Devilee 2012). The role of the M2 isoform of pyruvate kinase (PK) is debated, since it seems not to be a tumour-specific isoform(Bluemlein, Gruning et al. 2011) but is still of importance(Zhou, Li et al. 2012). The interaction between PK and HIF-1α is one example of interplay between metabolic pathways and those controlling cell growth (Luo, Hu et al. 2011).

Lactate dehydrogenase is key to a lactate-based signalling interaction between cancer and endothelial cells, which directly modifies endothelial cell function (Vegran, Boidot et al. 2011). Interestingly, these authors also demonstrated that lactate stimulated an NFκB/IL-8 pathway which resulted in cell migration and tube formation. This process was repressed by action of ROS inhibitors, suggesting their use by tumour cells as signalling molecules. Subsequent work by this group has indicated that p53-deficiency may permit increased expression of monocarboxylate transporter MCT1 (which transports lactate across the cell membrane) in hypoxic conditions(Boidot, Vegran et al. 2012). Loss of p53 alleviates inhibition of glycolysis with upregulation of glucose uptake transporters GLUT1 and GLUT4(Bensaad, Tsuruta et al. 2006).

p53 has also been implicated in the control of glutamine metabolism, whereby increased glutaminase activity reduces ROS levels and protects against ROS-mediated apoptosis(Suzuki, Tanaka et al. 2010). Loss of p53 as seen in most cancers will have complex effects, but as previously alluded to, increased levels of ROS may promote certain pathways, and create a pro-inflammatory state which facilitates tumour development.

1.9.3 Emphasis on biosynthesis

Cancer cells have high biosynthetic requirements to sustain their rate of proliferation(Icard and Lincet 2012). Lipid synthesis and the pentose phosphate

pathway, which provides the substrates for nucleic acid synthesis, are primarily supported. The glutamine pathway is important but it may be as a source of substrate for glycolysis rather than for protein synthesis. Amino acid usage far exceeds the rate of protein synthesis(Eagle, Oyama et al. 1956) and glutamine can be converted into lactate and α-ketoglutarate. Lactate, rather than being the end-product of the inefficient glycolytic pathway, may be of use to the tumour cell. Active acidification of the microenvironment may facilitate tissue invasion (Gatenby and Gawlinski 2003).

1.9.4 Redox status in cancer cells

Intrinsic and extrinsic redox buffer systems exist in all cells, and have diverse functions in signalling and modulation of cell fate, due to interaction with other apoptotic regulators such as NFκB (McEligot, Yang et al. 2005). Active acidification of their environment suggests that tumour cells are not subject to the normal mechanisms maintaining homeostasis in the cell and its surroundings, but tumour cells do require a plentiful capacity for oxidation in multiple synthetic reactions, most importantly in the form of NAD+. Conversion of pyruvate to lactate in the final step of glycolysis provides one source of this co-factor. Cancer cells are particularly prone to DNA breaks and cells seem to be particularly vulnerable to NAD+ usage by PARP in the response to such damage(Mazurek and Eigenbrodt 2003). NAD will be further discussed in the context of regulation of sirtuin function, and the functions of these potential biomarkers of ageing in colorectal cancer.

1.9.5 Inflammation and proliferation

NFκB, a mediator of inflammation, which has been mentioned above in conjunction with angiogenesis and lactate production, also coordinates cellular metabolism during proliferation by altering the balance between glycolysis and oxidative phosphorylation, promoting increased mitochondrial activity(Mauro, Leow et al. 2011). Inhibition of NFκB causes reversion to glycolysis under basal conditions, with necrosis of cells in case of glucose starvation. Cells lacking a subunit of NFκB showed characteristics of transformation such as anchorage-independent growth, even in the presence of wild-type p53. Although NFκB has been shown in some conditions to promote tumorigenesis through the facilitating

effect of its proinflammatory actions, it appears, like many other genes of central importance, to have dichotomous effects depending on context, tissue and contemporaneous state of activity of associated genes.

1.10 Biomarkers of Ageing

It will be clear from the preceding discussion that ageing, while it bears some relationship to chronological time since birth of an organism, is a complex process and cannot be accurately measured in terms of the period since birth. Instead, other markers have been sought for several years. Characteristics of these 'biomarkers of ageing" were defined as follows(Baker and Sprott 1988):

"A biomarker of aging is 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."

As yet no single biomarker, or composite panel, has been described that will definitively fulfil the criteria. It is recognised that the original definition invokes prerequisites that are difficult to fulfil such as separation of characteristics of the ageing process from disease processes. These may be of secondary importance in practical and clinical usage, when prognosis of functional capacity may be as useful as prognosis related specifically to disease progression. As such the concept of 'biomarkers of ageing and disease' has gained ground, particularly among some of the authors of the original definition(Sprott 2010).

1.10.1 Biomarkers of Ageing – level of measurement

Like senescence, useful measurement of ageing can be undertaken on several levels and candidate biomarkers may reflect this. Ageing of the whole organism may best be assessed with serum markers which integrate ageing from several systems.

More specific reflection of ageing in individual systems may be obtained from analysis of function within those tissues. Potential biomarkers of ageing may originate within the domain of metabolic control given the fundamental importance of the control of energy usage for the whole organism and on the level of tissue and cellular function.

The MTR hypothesis proposes that optimal control of cellular metabolism is achieved by the integration of function of the trinity of Mitochondria, Telomeres and Ribosomes(Shiels and Davies 2004). This is particularly important in response to any insult causing damage to the cell. Telomeres have an important role in limiting cell division if DNA damage is severe to prevent promulgation of dangerous mutations. Mitochondria assess and provide the necessary energy from respiration to fuel repairs. Ribosomal synthesis of the enzymes needed to perform the repairs progresses if this is a viable option. Failure of any one component of the trinity to respond adequately could be fed back to the others and instigate a switch to a safe default outcome of programmed cell death.

The sirtuins as a group provide an intuitive physical representation of this hypothesis, both because of their dependence on the redox state of the cell to function and because of their subcellular locations, which encompass all three of the key intracellular structures involved (nucleus, mitochondria and ribosomes).

Fetuin A is a negative acute phase protein with several biochemical functions including inhibition of TGFβ, calcium-binding capacity, and interactions with the insulin receptor, annexins and matrix metalloproteinases. As a serum protein, it may be a candidate biomarker of ageing at a whole-organism level.

These three subjects of study in this thesis have gained prominence in the fields of ageing research and also in the study of other human diseases. This suggests that as biomarkers of ageing or biomarkers of pathology and disease, they may be of importance in some of the fundamental pathways of regulation within human cells.

1.11 Fetuin A

There is a large body of work indicating the importance of fetuin A in a range of pathways (cell-cell interaction, inflammation, calcium physiology and metabolic regulation as well as cancer) but it is relatively dispersed among specialty areas. A comprehensive analysis of the literature was undertaken to achieve a fuller understanding of the breadth of its activity and interactions and to contextualise the experimental observations made on its associations with clinicopathological factors in colorectal cancer. A synopsis of relevant papers is included.

1.11.1 Discovery and naming

Fetuin was discovered in 1944 (Pedersen 1944), as a glycoprotein isolated from fetal calf serum. Subsequently α-2-Heremans-Schmid glycoprotein, purified in 1960 by Heremans(Heremans 1960) and Schmid(Schmid and Burgi 1961), was proved by similarity of structure to be identical to the human homologue of fetuin, referred to as fetuin A (Christie, Dziegielewska et al. 1987; Dziegielewska, Mollgard et al. 1987). Literature review is complicated by regular usage of both names, and there is a variety of spellings and punctuation of the composite name. There is additionally a human fetuin B protein, a separate member of the family about which even less is known(Olivier, Soury et al. 2000; Denecke, Graber et al. 2003).

1.11.2 Genetic location of fetuin A

Fetuin A is a single copy gene localised to chromosome 3q27(Magnuson, McCombs et al. 1988). This area is known to be susceptible to chromosomal translocations in non-Hodgkin's lymphoma (Bastard, Tilly et al. 1992) and the fetuin A gene is positioned in the same linkage group as transferrin, transferrin-receptor, and caeruloplasmin, and close to other members of the cystatin superfamily, of which it is a member.

The common fetuin A alleles are associated with serum levels of the protein, in a dose-dependent manner, such that levels are lower in allele 2 homozygotes, highest in allele 1 homozygotes and intermediate in heterozygotes(Osawa, Tian et al. 2005)

1.11.3 Protein structure of fetuin A

The mature form of the protein consists of two peptide chains, 'heavy' and 'light'. Twelve cysteine residues form 6 covalent disulphide bonds contributing to the protein's tertiary structure. The heavy chain contains two homologous cystatin domains (Elzanowski, Barker et al. 1988), indicating this protein's membership of the cystatin superfamily, although they do not have cysteine protease inhibitor capacity. The cystatin domains contain a calcium-binding motif(Heiss, DuChesne et al. 2003), and a TGFβ binding motif, with homology to the TGFβII receptor.

The third domain of fetuin, located on the 'light' chain, has a relatively hydrophobic character and contains stretches that resemble a transmembrane sequence. A degree of sequence homology with steroid receptor and growth factor receptors led to speculation that fetuin A may have a role of this sort (Dziegielewska and Brown 1995) (pp 82-84). Fetuin A is reported to exist in serum with 20% phosphorylation at steady state (0.2mol PO_4/mol fetuin A) (Haglund, Ek et al. 2001). Phosphorylated fetuin A alters function of the insulin receptor in humans and rats (Srinivas, Wagner et al. 1993; Mathews, Chellam et al. 2000). There are 5 sugar moieties attached to the human fetuin protein, 2 N-linked chains and 3 O-linked chains (Gejyo, Chang et al. 1983; Yoshioka, Gejyo et al. 1986; Hayase, Rice et al. 1992; Watzlawick, Walsh et al. 1992).

1.11.4 Genetic modulation of Fetuin A

1.11.4.1 Regulation of Fetuin A

The 5' transcription control region of the human and rat fetuin A gene contains binding sites for the promoters CCAAT enhancer binding protein (C/EBP) and NF-1 in the order 5'-N2-C2-N1-C1-3' and these are necessary for basal activation (Gangneux, Daveau et al. 2003).

IL1β and IL6 downregulate fetuin A in humans(Daveau, Christian et al. 1988). In a hepatoma cell line model, maximal decrease in fetuin A synthesis and secretion was observed after 4 days, despite incubation for 10 days with monocyte-conditioned medium (Hiron, Daveau et al. 1992). Macrophages produce TNFα, IL1 and IL6, although this paper did not specify an interaction with these cytokines.

Although IL6 acts primarily through the STAT pathway, one of its effects is to upregulate C/EBP synthesis in the liver. IL1β also transduces its effects through the C/EBP gene family. This may be why maximal downregulation of fetuin A requires both IL1β and IL6 input(Daveau, Christian et al. 1988). The initial report on the fetuin A promoter region suggested that there were potential binding sites for several other nuclear factors including STAT3(Banine, Gangneux et al. 1998). Overexpression of c-Jun and c-Fos also inhibits fetuin A gene transcription(Inoue, Takata et al. 2008).

Decrease in fetuin A mRNA transcription occurs in response to glutamine enrichment of the extracellular space and the resultant hypotonic stress (Claeyssens, Banine et al. 1998).

There is limited information on the upregulation of fetuin A but dexamethasone does have this effect. This interaction is mediated through C/EBP binding and hepatocyte nuclear factor-3 (HNF-3, which is a promoter predicted in earlier reports) (Woltje, Tschoke et al. 2006). Dexamethasone is a glucocorticoid, produced in response to the acute phase response and forms a negative feedback loop, inhibiting expression of proinflammatory mediators and resolving the episode of inflammation.

Regulation of fetuin A expression thus appears to be closely integrated with the control of the inflammatory response, and it is not surprising that fetuin A was initially studied as a negative acute phase protein.

1.11.5 Tissue localisation

1.11.5.1 Synthesis

There is limited information available about expression of fetuin A in human foetal tissues, but it is seen in osteoid material and lung tissues(Dziegielewska, Mollgard et al. 1987). Fetuin A is produced in adult humans by the liver and secreted into the bloodstream. Fetuin A mRNA has been reported in human kidney in the epithelial cells of distal and proximal renal tubule cells(Umekawa, Iguchi et al. 1999), and in the tongue and placenta(Denecke, Graber et al. 2003).

1.11.5.2 Plasma protein: variation in levels in foetus and adult

Fetuin A is secreted from the liver and is present at higher levels in the human foetus where it forms approximately 5.8% of plasma protein. This decreases to 0.6% of plasma protein in adults(Dziegielewska, Mollgard et al. 1987). There is evidence of significant variation among individuals, but serum levels of fetuin A of approximately 100mg/100ml are standard in newborn humans, and fall to around 60mg/100ml in adulthood. Lower levels observed in disease are of the order of 30-40mg/100ml(Dziegielewska, Guminska et al. 1993).

Interestingly, fetuin A is one of a handful of proteins tested which show higher serum levels in women than men, decrease after menopause and are restored to premenopausal levels by administration of exogenous oestrogen(Hashimoto, Miwa et al. 1991). This may be of relevance to fetuin A as a biomarker of ageing in that it parallels the change in telomere length in PBLs. PBL telomere lengths are generally longer in women, but this advantage is lost after menopause.

1.11.5.3 Target tissues for the secreted protein in the adult

Immunohistochemistry of human colorectal tissue and tumour samples has shown extracellular staining consistent with fetuin A binding to the cell membrane (Swallow, Partridge et al. 2004), with lower staining in tumour tissue. Potential associations with bone mineral density and osteoporosis, another age-related disease, are discussed later in the context of fetuin A and calcium metabolism, implying bone as another target tissue.

1.11.6 Fetuin A in Inflammation and the Acute Phase Response

1.11.6.1 Fetuin A – a negative acute phase protein

Initial studies identified fetuin A as a negative acute phase protein (whose levels decrease in response to inflammation) in illnesses of varying aetiology including trauma(van Oss, Bronson et al. 1975), bacterial infection(Lebreton, Joisel et al. 1979), Crohn's disease(Kalabay, Cseh et al. 1992) and systemic lupus erythematosus(Kalabay, Jakab et al. 1990) and in cancer(Baskies, Chretien et al. 1980). All of these are now recognised to have a significant inflammatory component.

In cattle and mice fetuin is a positive acute phase protein, and a negative acute phase protein in rat. This distinction underlines the care that must be taken in translating results between species even in evolutionarily conserved proteins: the molecular pathways may have significantly diverged.

Modulation of fetuin A in the short term acute phase response to conditions like bacterial infection shows some differences from that observed in the longer term response to multiple myeloma(Biro, Domjan et al. 1998). Myeloma patients showed correlation between fetuin A and TNFα but not with IL6, whereas CRP correlated with IL6 in both conditions.

1.11.6.2 Fetuin A and the innate immune system. Opsonin, increases uptake of inhibitors of cytokine synthesis

Much initial information from human studies concentrated on the interaction of fetuin A with the several classes of blood cells involved in the innate immune response. Fetuin A enhanced phagocytosis of E coli and S aureus by neutrophils(van Oss, Gillman et al. 1974). Fetuin A improved the rate of uptake of DNA and latex beads by macrophages and monocytes from mouse and human blood(Lewis and Andre 1981). It appeared not to function as a classic opsonin as it was necessary for the phagocyte, fetuin A and the target to be present at the same time for the enhanced phagocytosis to be demonstrated. Fetuin A has also been shown to be chemotactic for monocytes but not neutrophils or lymphocytes(Malone and Richards 1987; Kew and Webster 1988).

Binding of fetuin A to macrophages was partially calcium dependant and resulted in some internalization of the protein(Lewis and Andre 1981). Internalization is achieved through membrane-ruffling and macropinocytosis (Jersmann, Dransfield et al. 2003). One model to integrate these reports is that fetuin A binds opsonic targets and then binds to a cell-membrane receptor which stimulates uptake. Given the repertoire of receptor binding exhibited by fetuin A, there are several possible candidates. Cell-surface receptors are discussed in more detail in the following sections.

The role played by fetuin A as a non-classical opsonin has been implicated in the resolution of the inflammatory response. Macrophages are activated by proinflammatory cytokines, NO and LPS, among other substances. Balancing this are anti-inflammatory cytokines such as TGFβ, IL10 and glucocorticoid hormones. Spermine is a ubiquitous polyamine that accumulates at sites of injury, and is deacetylated by sirtuins (see section 1.15) (Grozinger 2002). Spermine uptake reduces macrophage production of TNFα, IL1 and IL6 (Zhang,

Caragine et al. 1997). Fetuin A binds spermine and the complex inhibits macrophage secretion of TNFα.

Macrophages use fetuin A to take up CNI-1493, a pharmacological cationic inhibitor of cytokine synthesis from the extracellular environment. They respond by a downregulation of TNFα synthesis (Wang, Zhang et al. 1998). It was not clear how the intracellular transport was mediated. Given that the C-terminal part of fetuin A has a negative charge, addition of a positively charged molecule of endogenous (spermine) or synthetic (CNI-1493) origin may render it more lipophilic and facilitate transport across the cell membrane. The process of membrane-ruffling and pinocytosis described above may be involved.

TNFα has a major role to play in the immune response underlying rejection of transplanted (foreign) tissue. During pregnancy, rejection of 'foreign' foetal tissue must be prevented. Levels of spermine and fetuin A are particularly high in the amnion. Thus, fetuin A may help maintain the privileged immune status of the foetus (Wang, Zhang et al. 1997).

Thus, fetuin A has a role in deactivating macrophages by increasing spermine uptake but also inhibits TGFβ, an anti-inflammatory cytokine. Its actions in this area (as in many others) are not amenable to simple dichotomous description.

1.11.7 Fetuin A and inhibition of TGFβ signalling

One of the fetuin A cystatin domains includes a motif with homology to the TGFβ2 receptor which allows fetuin to bind several of the TGFβ family with reducing affinity as follows: BMP2 > BMP4 > BMP6 > TGFβ1 > TGFβ2 (Demetriou, Binkert et al. 1996).

1.11.7.1 TGFβ signalling and cancer

As has been previously mentioned TGFβ has several important roles relevant to cancer pathophysiology. It inhibits phosphorylation of Rb which supports cell-cycle arrest in G1 phase (Laiho, DeCaprio et al. 1990). It has been identified as a trigger for induction of senescence in cultured cell lines (Katakura 2006). Cells senesced in this manner showed reduced tumorigenic potential. It is also

instrumental in the process by which haematopoietic stem cells are recruited to supply the stem cell niche in the gut(Sipos, Valcz et al. 2012).

TGFβ is also a strong driver for epithelial-to-mesenchymal transition (EMT), which alters cellular interaction with the microenvironment (Cufi, Vazquez-Martin et al. 2010). TGFβ-induced EMT increases cellular motility and increases 'stemness': replicative potential and de-differentiation. TGFβ can thus be seen to have cytostatic (tumour suppressive) and oncogenic effects (Elliott and Blobe 2005). Ultimately the effect it has at any one time is likely to be determined by the genetic context, other mutations and the cellular microenvironment: a recurring theme in cancer biology (Schiemann 2007).

TGFβ has anti-inflammatory cytokine properties and reduced activity may be a factor in the development of IBD and in the increased risk of cancer these patients show (Skeen, Paterson et al. 2012). With regard to risk of developing sporadic colorectal cancer, polymorphisms in the TGFβ gene and in the TGFβ receptor are associated with increased risk, and interaction with NFκB signalling and lifestyle factors such as obesity, aspirin use and smoking were also demonstrated(Slattery, Lundgreen et al. 2012). Further analysis of high-risk genotypes has shown a hazard ratio for survival after diagnosis with colorectal cancer of 5.1 for the group with the highest number of 'at risk' genotypes(Slattery, Lundgreen et al. 2011). This group has also shown high-risk genetic variation in BMP proteins, which may also be relevant to fetuin A interactions in colorectal cancer(Slattery, Lundgreen et al. 2012).

1.11.7.2 Fetuin A, TGFβ signalling and cancer

Inhibition of TGFβ signalling by serum fetuin A in cell lines has been demonstrated(Swallow, Partridge et al. 2004). Fetuin A blocked TGFβ binding to its receptor on MvLu1 cells, preventing intracellular signal transduction and EMT effects. This team also showed by immunohistochemical analysis that fetuin A binding to human colorectal tumour tissue was reduced to one third of the binding to normal colorectal mucosa. The overall effect of fetuin A on the pathogenesis of colorectal cancer may therefore be as variable as that of TGFβ since it opposes some of the oncogenic potential of TGFβ but appears to bind less to tumour than to normal tissue.

Further information on the dynamics of fetuin A interaction with TGFβ in serum matched with an assessment of fetuin and TGFβ binding to colorectal normal and tumour tissue may add useful information. Given that fetuin A also binds to other cell surface receptors (e.g. annexins in breast cancer(Sakwe, Koumangoye et al. 2010)) it would be interesting to know which receptors fetuin A is interacting with on the colorectal cell surface. Since fetuin A has also been shown to inhibit TGFβ signalling in a mouse model of mammary tumourigenesis, it is possible that both pathways are active in colorectal cancer too (Guillory, Sakwe et al. 2010).

1.11.8 Fetuin A in calcium and phosphate metabolism

The inhibition of TGFβ family members, particularly modulation of BMP cytokines, indicated a role for fetuin A in embryological skeletal modelling and potentially in bone remodelling by osteoblasts. Fetuin A was described in early reports as an inhibitor of ectopic calcification and was unusual in that it was the first circulating molecule with such action described.

Fetuin A is one of a small number of non-collagen plasma proteins identified in bone, and is present there at much higher concentrations than in plasma. Comparison of levels at different ages and in different pathologies suggested likely involvement in bone turnover and resorption(Quelch, Cole et al. 1984).

Fetuin A was also identified in ascites fluid in patients with cancer, when it was demonstrated that it was one tenth as effective as parathyroid hormone at releasing calcium from bone (Lamkin, Colclasure et al. 1987). This effect on calcium was seen at lower concentrations. In fact, in vitro, fetuin A has a variable effect. Calcium mobilisation is higher at lower concentrations, peaking at serum fetuin A level of 40 micrograms/ml and returning to baseline at (Colclasure, Lloyd et al. 1988). Of note, this threshold is equivalent to 0.04mg/ml, well below the ranges reported in normal or diseased states but may be relevant in terms of local fetuin A concentrations in bone (Dziegielewska, Guminska et al. 1993).

1.11.8.1 Fetuin A and osteoporosis in postmenopausal women

As fetuin A is a constituent of calcified bone and intimately involved in calcium metabolism, studies of bone mineral density in postmenopausal women were performed to investigate an association with osteoporosis, another age-related disease. There is a significant dose-dependent association between fetuin A polymorphisms and oestradiol levels(Eichner, Friedrich et al. 1990). Homozygotes for allele 1 showed lowest levels of oestradiol, homozygotes for allele 2 showed highest levels and heterozygotes showed intermediate levels.

1.11.8.2 Action of fetuin A in prevention of ectopic mineralisation and inflammation

Serum is supersaturated with calcium ions and the natural tendency is for ectopic mineralisation to occur, unless the calcium ions are sequestered and stabilized in some form. Initial studies found that fetuin A bound basic calcium phosphate crystals, and reduced the hydroxyapatite crystal-induced release of superoxide from neutrophils(Terkeltaub, Santoro et al. 1988).

“Calciprotein particles” are colloidal spheres, similar to the apolipoprotein conglomerations which allow the transport of insoluble lipids through the bloodstream (Heiss, DuChesne et al. 2003). The calciprotein particles are transiently soluble and prevent formation of basic calcium phosphate (precursor to apatite) for 6 hours.

Formation of these particles could reduce the local inflammatory effect of crystals, and thus allow a coordinated degradation, without triggering the non-specific immune response. This may include targeted recruitment of phagocytes which can digest the calciprotein particles. High molecular weight complexes of calcium, phosphate, fetuin A and matrix GLA protein were described recently (Jahnen-Dechent, Heiss et al. 2011). They protect against the otherwise chemically favourable tendency to mineral deposition in most tissues where it is undesirable and results in inflammation.

1.11.9 Fetuin A and role in Vascular Calcification

Vascular disease is recognised as having a significant inflammatory component. Deposition of calcium in atheromatous plaques is a large part of the pathogenesis of this disease and it is not surprising that fetuin A has attracted

interest in this field. Initial studies confirmed presence of fetuin A in aortic atheromatous plaques(Keeley and Sitarz 1985; Stastny, Fosslien et al. 1986)

Fetuin A is inversely correlated with calcification in patients with peripheral vascular disease but without renal disease, and this relationship persists on adjusting for traditional risk factors such as diabetes(Szeberin, Fehervari et al. 2011).

Fetuin A inhibits vascular smooth muscle cell (VSMC) calcification through inhibition of apoptosis and caspase cleavage (Reynolds, Skepper et al. 2005). Fetuin A is taken up by VSMCs and concentrated in intracellular vesicles. These are subsequently released. Vesicles have previously been shown to take up basic calcium phosphate and were a focal point for mineralisation. Presence of fetuin A prevents their nucleation of the mineral ions and increases vesicle reuptake.

1.11.10 Fetuin A and renal disease

Calcium handling in renal disease is altered because the damaged kidney cannot play its role in normal calcium homeostasis. Patients with chronic renal failure have an increased risk of death from cardiovascular causes. Fetuin A is one nexus for the processes of inflammation, altered calcium handling and vascular disease which underlie the pathological manifestations of chronic renal failure (Tsirpanlis 2007). IL6 as an inflammatory cytokine could connect inflammation and cellular senescence in this context, in a pathway which is likely to involve fetuin A (Tsirpanlis 2009). Low level IL6 as seen in patients with end stage renal disease but no signs of systemic inflammation does downregulate hepatic synthesis of fetuin A(Memoli, Salerno et al. 2010).

Low fetuin A has been linked to all cause mortality (Carrero, Stenvinkel et al. 2008) and cardiovascular mortality in renal patients irrespective of CT-assessed coronary vascular calcification(Jung, Baek et al. 2011).

1.11.11 Fetuin A as an inhibitor of the insulin receptor tyrosine kinase

Fetuin A inhibits the tyrosine kinase activity of the insulin receptor(Srinivas, Wagner et al. 1993). In rat and cow, the phosphorylated form of fetuin is a

single chain protein, and it is the phosphorylated form which exhibits activity against the insulin receptor(Haasemann, Nawratil et al. 1991). There is no direct experimental demonstration that the same is true in humans, but the phosphorylated form of human fetuin A is separately metabolically active. After a bout of exercise, phosphorylated fetuin A, but not total fetuin A, levels were reduced in lean individuals(Mathews, Grandjean et al. 2010). The recombinant single chain form of human fetuin A has markedly greater activity on the insulin receptor tyrosine kinase than does the form isolated from human plasma(Srinivas, Deutsch et al. 1996).

Binding of insulin to the insulin receptor induces a conformational change which enhances fetuin A binding; one of the results is to inhibit insulin-stimulated autophosphorylation of the insulin receptor(Mathews, Chellam et al. 2000). This interaction does not alter insulin binding to the receptor but does markedly reduce insulin-stimulated GLUT4 translocation and AKT activation in mouse myoblasts(Goustin, Derar et al. 2013).

Fetuin A abrogates the mitogenic effects of insulin in H-35 hepatoma cells (Srinivas, Wagner et al. 1993). Through this mechanism, fetuin A inhibits insulin-induced activation of Ras signalling in Chinese hamster ovary cells overexpressing the human insulin-receptor(Srinivas, Deutsch et al. 1996). In this system, RAF phosphorylation was also reduced with resultant abolition of MEK phosphorylation; phosphorylation of SHC was abolished as well. RAF is part of the RAS/RAF/MEK/ERK signalling pathway and SHC is part of the stress-induced ERK activation response.

A polymorphism of the fetuin A gene in humans associated with lower serum levels is associated with leanness in a study of 504 Swedish men(Lavebratt, Wahlqvist et al. 2005). Fetuin A is thus characterised as a ‘thrifty gene’, which blunts the response to insulin, tending to tip the balance of metabolism towards storage rather than cell division (Goustin and Abou-Samra 2011).

1.11.12 Fetuin A: role in metabolic regulation and diabetes

Given the alteration in sensitivity to insulin, associations with Type 2 diabetes have been a focus for research. Fetuin A was associated with insulin sensitivity

in normoglycaemic but not diabetic subjects (Mori, Emoto et al. 2006). In non-diabetic Caucasians, fetuin A levels were higher in those with reduced insulin sensitivity and impaired glucose tolerance and were correlated with liver fat(Stefan, Hennige et al. 2006). Fetuin A was higher in the vitreous humour of patients with diabetic eye disease than those with macular degeneration(Nakanishi, Koyama et al. 2002). Fetuin A polymorphisms have been associated with diabetes in Danish and French populations (Siddiq, Lepretre et al. 2005; Andersen, Burgdorf et al. 2008).

Fetuin A is associated with liver steatosis and general BMI but not with regional fat distribution in humans(Mussig, Staiger et al. 2009). Allelic variation in the fetuin A gene alters the effect of exercise on change in basal metabolic rate (Suchanek, Kralova-Lesna et al. 2011). Some variants were associated with increase in lean muscle mass and decrease in total body fat, but there was no significant association with BMI.

Diabetes is one of the traditional risk factors for cardiovascular disease. Fetuin A levels were compared in diabetic patients with and without peripheral vascular disease (PVD) and in patients with normal glucose metabolism and PVD: fetuin A levels were higher in patient with PVD irrespective of their glucose metabolism(Lorant, Grujicic et al. 2011). It is possible that vascular disease precedes diabetes, occurring through the same dysfunctional response to insulin and dysregulation of glucose metabolism, particularly in the presence of longterm excess caloric intake.

1.11.13 Fetuin A and Cancer

1.11.13.1 Relevance of previously discussed molecular interactions

The molecular interactions of fetuin A described earlier are relevant to cancer biology in several ways.

As an opsonin, fetuin A may modulate the innate immune response to the tumour. By its effect on TNFα levels, this may affect the rejection of abnormal cells.

Calcium regulation is important for signalling within cells, so management of external cell calcium homeostasis may be relevant. Prevention of abnormal calcification in damaged tissue and coordination of clearing of calcium-containing crystals would reduce the local inflammatory response in the tumour environment.

As an inhibitor of TGFβ, fetuin A may modify transcription of a wide variety of genes important in cell death, differentiation and the response to injury which has been shown to contribute to the malignant phenotype. It also provides another modulatory role for fetuin A in calcium regulation, as this is another area in which TGFβ is effective. Fetuin A is a negative APP which inhibits TGFβ which in turn has anti-inflammatory properties: fetuin A is intimately linked to the process of inflammation, but may be more affected than an effector of the process. IL6 regulation of fetuin A implies it may be a target in ameliorating the poor prognostic impact of high IL6 levels and active systemic inflammation in cancer.

Matrix metalloproteinases (MMP2 and MMP9) are targets for fetuin A, and these are important in cell interaction with the extracellular matrix. The gelatinase activity of MMP9, protected by fetuin A, may increase cell motility(Ochieng and Green 1996).

Inhibition of tyrosine kinase activity of the insulin-receptor means that fetuin A plays a role in metabolic regulation, which has recently become a focus for attention as it is profoundly altered in cancer cells.

The interaction with Annexin 6 will be discussed in detail later but is included in this summary of molecular interactions (Sakwe, Koumangoye et al. 2011)for completeness. Fetuin A binds to Annexin 6 with effects on cell-to-cell adhesion and mobility, relevant in the metastatic behaviour of breast cancer cells and conceivably the cells of other cancers.

All of these are viable reasons why fetuin A should be a relevant target in treating cancer, and they are strengthened by the following reports of alteration in fetuin A levels in cancer and a relationship with survival in one type of cancer shown so far.

1.11.13.2 Altered serum levels in cancer

There is a wide-ranging literature attesting to altered fetuin A levels in cancer. Most reports show decreased serum levels of fetuin A, in line with its recognised role as a negative acute phase protein, altered within the acute phase response which occurs specifically to cancer. Control of fetuin A levels appears to be similar in cancer and in shorter infective illnesses, in contrast to CRP (Biro, Domjan et al. 1998). It is not certain therefore that the poor prognostic value of high CRP will be associated with alteration in fetuin A levels as well.

The change in fetuin A levels in cancer may be part of the body's inflammatory response to cancer. Equally however, the tumour cell handling of fetuin A which binds to it may subvert this response.

Reduced levels of fetuin A (relative to normal) have been demonstrated in serum in haematological malignancies including acute myeloblastic leukaemia(Wiedermann, Wiedermann et al. 1980; Kalabay, Cseh et al. 1991; Kwak, Ma et al. 2004), germ-line ovarian carcinoma(Chen, Lim et al. 2008), cervical cancer(Onizuka, Migita et al. 1994) and in squamous cell carcinoma of the lung (Dowling, O'Driscoll et al. 2007). Barbieri et al reported that fetuin A levels discriminated between patients with and without bony metastases (Barbieri, Pazzaglia et al. 1987).

Reduced levels of fetuin A (relative to normal) have also been demonstrated in nipple aspirate in breast cancer(Pawlik, Hawke et al. 2006).

1.11.13.3 Serum fetuin A and cancer survival

Patients with normal levels of fetuin A in serum with glioblastoma had longer survival than those with low levels (Petrik, Saadoun et al. 2008). However, levels of two isoforms of fetuin A in CSF were higher in patients with low-grade gliomas than in CSF from non-neoplastic or normal cases(Ribom, Westman-Brinkmalm et al. 2003). Brain is one of few other suggested sites of fetuin expression, although it has not been confirmed in the adult (Dziegielewska and Brown 1995). CSF fetuin A levels may thus reflect cancer cell synthesis beyond the blood-brain barrier rather than serum levels and the host response to the cancer.

Fetuin A levels in multiple myeloma correlated inversely with serum calcium, and showed significant decrease from relatively stable levels as calcium rose in the end stages of disease(Crawford 1984).

1.11.13.4 Fetuin A in liver disease and liver cancer

Serum fetuin A levels were raised in hepatocellular carcinoma (HCC) (Wang et al. Cancer Letters (2009) 281:144–150). Fetuin A is synthesised by hepatocytes, so increased expression in carcinoma of hepatocellular origin may reflect tumour cell phenotype. Levels of fetuin A decreased after radiofrequency ablation of HCC lesions(Kawakami, Hoshida et al. 2005).

1.11.13.5 Fetuin A and lung cancer

Glycoproteomic analysis of human lung adenocarcinoma tissue showed 30 differentially expressed proteins, of which only fetuin A showed concordant decrease in mRNA and protein levels. The glycan structure of fetuin A in lung tissue also showed alterations in tumour tissue, with high mannose component not seen in fetuin A from normal tissue(Rho, Roehrl et al. 2009).

1.11.13.6 Fetuin A and breast cancer

There is a significant body of work by Ochieng and colleagues investigating fetuin A in breast cancer. They have shown that breast cancer cells interact with serum-derived fetuin A by binding to cell-surface Annexin-6 in a calcium dependent manner(Ochieng, Pratap et al. 2009; Sakwe, Koumangoye et al. 2010). This interaction stimulates PI3K and AKT; it also activates MAPK independently of calcium. Annexin-6 is involved in cell-cell interaction and mouse mammary tumour lines with Annexin-6 knockout show increased anchorage independent growth, which may indicate the tendency to metastasize(Guillory, Sakwe et al. 2010).

Fetuin A and antibodies to it were identified as potential tumour biomarkers in breast cancer; the sensitivity of fetuin A antibodies was 79%, which may not be sufficiently high(Yi, Chang et al. 2009). It may be useful as part of a panel of biomarkers and several investigators have proposed this(Schaub, Jones et al. 2009). In HCC, a panel of fetuin A, KRT23 and ferritin light chain antibodies had

sensitivity of 98.2% in detection of HCC over chronic hepatitis and normal sera(Wang, Xu et al. 2009).

1.11.13.7 Fetuin A and colorectal cancer

Blockage of TGFβ signalling by fetuin A has been shown to prevent phosphorylation of Smad 2/3 in a mouse cell line and blocks epithelial-mesenchymal transition of colorectal cancer cells (Swallow, Partridge et al. 2004). Immunohistochemical analysis demonstrated specific reduction of fetuin A in colorectal tumour tissue as compared with adjacent normal mucosa.

Recent evidence indicates that there is interplay between IL6, STAT3 and fetuin A in chronic renal disease(Memoli, Salerno et al. 2010). The IL6-JAK-STAT3 pathway is important in colonic tumorigenesis by bone-marrow-derived myofibroblasts (Zhu, Cheng et al. 2014) and this network also involves TGFβ. Fetuin A is likely to have a role in this process also.

Familial adenomatous polyposis (FAP) is associated with almost 100% risk of colorectal cancer by the 4th decade of life. Proteomic analysis of sera from patients with (FAP) identified fetuin A and apolipoprotein D as significantly downregulated in carpeting FAP with respect to the diffuse form, and to normal serum(Quaresima, Crugliano et al. 2008).

Fetuin A is a TGFβ antagonist, with effects on BMP (members of the same family). BMPs exert growth inhibitory effect towards the top of crypts which renew the colonic epithelium(Zeki, Graham et al. 2011). Fetuin A inhibition of BMP may increase the chance of aberrant proliferation of colonic cells. However, the available evidence shows decreased binding of fetuin A to colonic epithelium in colonic cancer. Further information on the existence and location of interaction between fetuin A and BMP, and on the outcomes of such interaction are necessary to interpret the available information.

1.11.14 Biomarker of ageing

Fetuin A is associated with several diseases of ageing including diabetes, vascular disease in its many forms, Alzheimer's disease, osteoporosis and cancer. Unlikely as it may have seemed previously, an underlying inflammatory process

seems to be a common feature of all of these. Fetuin A may offer characteristics of a candidate integrative biomarker of ageing, but it is more likely that a composite measure of several factors will be necessary to adequately represent the dysfunction of such a complex process.

1.12 The Sirtuin family

The silent information regulator type 2 (SIR2) gene came to prominence in yeast, when it was noted that overexpression was associated with increased lifespan, and underexpression with shortened lifespan(Kaeberlein, McVey et al. 1999). Homologues for this gene family known as sirtuins have been identified in many other classes of organism including mammals, with similar effects on lifespan in mice. There are seven sirtuins (SIRT1-SIRT7) in humans. SIRT3 is associated with increased lifespan as will be discussed in more detail in the following sections on the individual sirtuins. In general terms, sirtuins seem to be particularly important in protecting internal systems from external environmental variation and stresses, maintaining systemic homeostasis and modulating more drastic responses to injury(Satoh, Stein et al. 2011).

1.12.1 General control of sirtuin activity: NAD

Sirtuins use nicotinamide adenine dinucleotide (NAD) as a co-factor in deacetylation reactions. Control of sirtuin activity is thus linked to cell metabolism and to the redox status of the cell. Control is both sirtuin-specific, and tissue specific, with activation and inhibition of different sirtuins in the same tissue. Part of the control is via NAD levels and thus the NAD cycle is of considerable importance. Different sirtuins have different dissociation constants for NAD (Schmidt, Smith et al. 2004), and this is particularly relevant for sirtuins operating in the same subcellular compartments, e.g. SIRT3, SIRT4 and SIRT5.

1.12.1.1 NAD cycle

There are two separate NAD pools within human cells: nuclear/cytoplasmic and mitochondrial, as NAD cannot pass across the mitochondrial membrane.

NAD levels are affected by the redox state of the relevant cell compartment but also by the activity of NAMPT, the rate-limiting enzyme in the NAD cycle. NAD is

used as a co-factor in enzymatic reactions by sirtuins (among many other enzymes) and nicotinamide is formed. This is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT). Regeneration of NAD is completed by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) which catalyses the addition of ATP to NMN (Imai and Yoshino 2013). NAMPT may regulate sirtuin activity by its effect on levels of NAD(Hufton, Moerkerk et al. 1999)

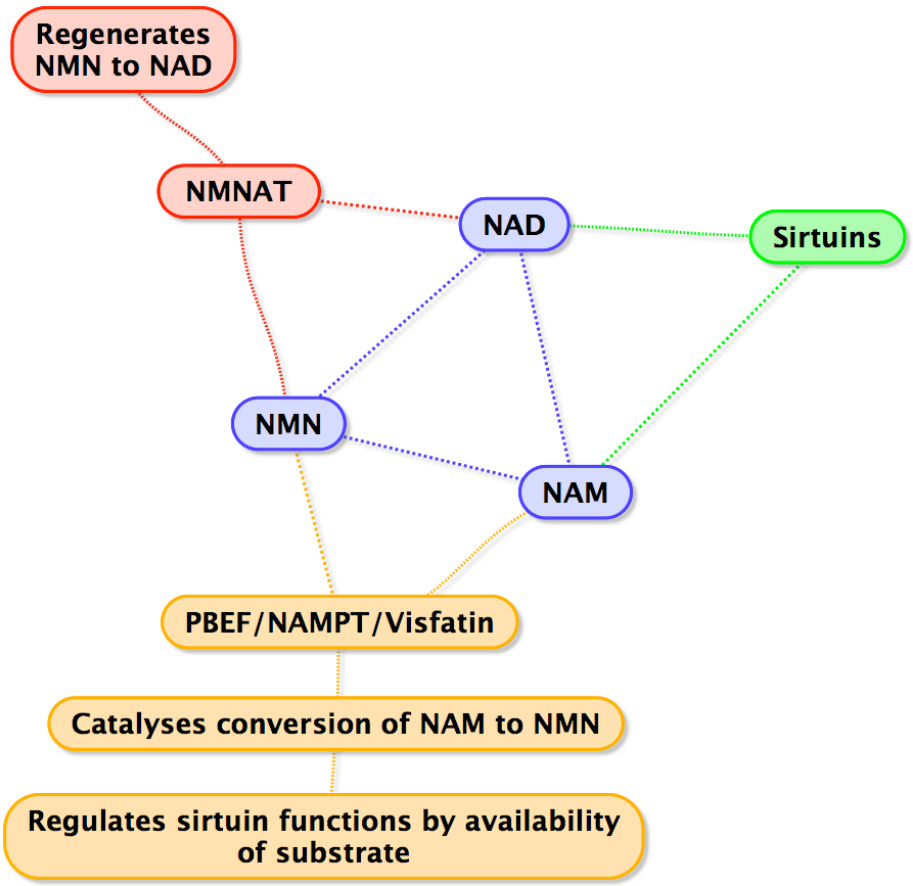

Figure 1.1 The NAD cycle (simplified). NAD is used as a co-factor in enzymatic reactions by sirtuins (among many other enzymes) and nicotinamide is formed. This is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) the rate-limiting enzyme. Regeneration of NAD is completed by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) which catalyses the addition of ATP to NMN.

1.12.1.2 NAMPT/PBEF

As well as being the rate-limiting enzyme in NAD synthesis, there is a growing body of evidence that NAMPT has diverse functions outside the cell as well (Luk, Malam et al. 2008; Imai 2009). One of the other names it was given on discovery

in microarray studies was pre-B cell colony-enhancing factor (PBEF)(Samal, Sun et al. 1994), and its cytokine functions include induction of TNFα, Il-1β and IL-6(Moschen, Kaser et al. 2007). It is also an adipokine with effects that both mimic and oppose those of insulin (Fukuhara, Matsuda et al. 2005). NAMPT has also been implicated in the genesis of colorectal carcinoma(Hufton, Moerkerk et al. 1999). Elucidation of its place in the complex regulatory strategy (on levels above the sirtuins) is likely to provide further fascinating insights into the precision of integration of organism-wide homeostatic systems.

Aspects of function specific to the individual sirtuins are discussed in the following sections, with a focus on the sirtuins investigated in this thesis. This is followed by consideration of how the sirtuins contributes to control of metabolism, inflammation, cell cycle and cancer development. Over half of the published literature on human sirtuins is focused on SIRT1 as it was the original sirtuin identified, and has the greatest homology to the yeast SIR2 gene. However, no new data on SIRT1 is presented in this thesis and so only a brief review of its important functions and interactions will be undertaken here.

1.13 SIRT1

The table below summarises major SIRT1 substrates and these interactions are considered in more detail in following paragraphs.

1.13.1 SIRT1 involvement in the DNA damage response

As the table at the beginning of this section indicates, SIRT1 numbers among its direct substrates four of the key players in the nucleotide excision repair pathway for dealing with single-strand DNA breaks. SIRT1 deacetylates and activates APE (Yamamori, DeRicco et al. 2010), NBS1 (Yuan and Seto 2007), XPA (Fan and Luo 2010) and XPC (Ming, Shea et al. 2010). The latter three are involved in sensing DNA damage and recruiting other repair proteins.

One of the original and consistently important interactions is between p53 and SIRT1. Deacetylation of p53 by SIRT1 inhibits p53-induced apoptosis. This is thought to protect cells from apoptosis in times of stress, as it allows a period for normal physiology to resume if possible.

Cell cycle control	Transcription	Signalling	Metabolism	DNA damage response	Other
p53	FOXO1, FOXO3a, and FOXO4, forkhead transcription factors	β-catenin, component of Wnt signaling pathway	Acetyl CoA synthetase 1, AceCS1	Ku70, initiates non-homologous end-joining (NHEJ)	BMAL1 and Per2, circadian rhythm/ clock proteins
PTEN, tumor suppressor regulating PI3K/ AKT pathway	PCAF, transcriptional coactivator associated with p53	RelA/p65 (NFκB component), immune function and signalling	PGC-1alphas, nuclear coactivator involved in control of gluconeogenic genes	Apurinic/ apyrimidinic endonuclease-1 (APE1), base-excision repair pathway	Cortactin, promotes cell migration
Notch1 intracellular domain (NICD), signalling pathway regulating cell fate	Myocyte enhancer factor, (MEF2), transcription factor	Oestrogen receptor-α	PPAR-gamma, nuclear receptor regulating fatty acid oxidation and glucose metabolism	p300 histone acetyltransferase, inflammation and DNA damage response	IRS-2, important for neuroprotection
p73, putative tumor suppressor involved in cell cycle, apoptosis	Hypoxia-inducible factors HIF-1alpha, HIF-2alpha	Androgen receptor	Uncoupling protein 2 (UCP2), inhibits glucose-stimulated insulin secretion	Nijmegen breakage syndrome protein (NBS1) part of MRE11-RAD50-NBS1 (MRN) complex	Tau, implicated in neurodegeneration and Alzheimer's disease
E2F1, transcription factor with role in cell cycle control	SREBP-1C, transcription factor modulating hepatic lipogenesis	Liver V receptor (LXR), nuclear hormone receptor		Xeroderma pigmentosum protein (XPA), DNA damage sensing and nucleotide excision repair	
	TAF(I)68, Pol I transcription	TSC2, part of the mTOR complex		Xeroderma pigmentosum protein C(XPC), as XPA	
	SUV39H1, histone methyltransferase	Rictor, part of the mTOR complex		TIP60, histone acetyltransferase, dsDNA break repair	
	TNF-alpha promoter	SMAD7, TGFβ-pathway			
	IL-1beta promoter				
	STAT3, transcription factor				

Table 1.1 Summary of important SIRT1 substrates.

1.14 SIRT2

1.14.1 Gene location and subcellular localisation: nuclear-cytoplasmic shuttling

The SIRT2 gene is localised to chromosome 19q13.1, and the 5' flanking region of DNA has binding sites for NFκB and GATA transcription factor(Voelter-Mahlknecht, Ho et al. 2005). A total of 16 exons are encoded. This group identified two isoforms of the protein translated: isoform 2 lacked the first 3 exons.

SIRT2 is found in the nucleus and in the cytoplasm in human cells. The SIRT2 gene encodes an N-terminus nuclear export signal but at least one human isoform does not contain this sequence of amino acids. Subcellular localisation and specific trafficking of SIRT2 protein are likely regulatory mechanisms. As yet no nuclear localisation sequence has been identified, so that it is unclear how the protein enters the nucleus.

It appears that nuclear export is the dominant process in non-dividing cells(North and Verdin 2007). SIRT2 is actively exported from the nucleus during interphase via a nuclear export signal which is leptomycin-B sensitive and interacts with Crm1(Inoue, Hiratsuka et al. 2007; North and Verdin 2007). There may also be a second nuclear export signal functioning independently of leptomycin-B, as a fraction of SIRT2 persists in the cytoplasm, possibly bound to structures there.

There is little information on which isoforms of SIRT2 are concerned in the interactions with other substrates and proteins described in the following paragraphs.

1.14.2 Interaction with tubulin

SIRT2 deacetylates lysine 40 of α-tubulin, mainly in the cytoplasm. It colocalises with HDAC6, another tubulin deacetylase(North, Marshall et al. 2003). α-tubulin binds only to the SIRT2/HDAC6 complex (Nahhas, Dryden et al. 2007). These investigators also showed that SIRT2 is enzymatically active in phosphorylated and unphosphorylated forms, and that cells which overexpress the shorter isoform2 of SIRT2 have reduced cell survival. Tubulin, which exists in at least 4

forms (α-δ) in humans(Smrzka, Delgehyr et al. 2000) is an integral part of microtubules, involved in cell mobility and in cytokinesis, when the single cell splits apart as the two daughter cells are formed in mitosis. In the formation of microtubules in the cytoskeleton to enable cell motility, tubulin is generally cytoplasmic in location, but as the nuclear membrane breaks down in mitosis, it is associated with chromosomes and other nuclear components.

SIRT2 induction reduces adhesion of cultured cells to substratum, in association with reduced levels of acetylation of α-tubulin(Pandithage, Lilischkis et al. 2008).

1.14.3 SIRT2 in mitosis

In dividing cells, the interaction between SIRT2 and α-tubulin is a key factor, and it was reported on the basis of experiments with microtubule poisons that SIRT2 functions in early metaphase to prevent chromosomal instability(Inoue, Hiratsuka et al. 2007). SIRT2 blocks entry into chromosome condensation in response to mitotic stress, implying that it acts as a mitotic checkpoint sensor, preventing progression of cell division before appropriate chromosomal alignment.

SIRT2 levels increase during mitosis and the protein is multiply phosphorylated during G2/M transition in the cell cycle. Wildtype overexpression mutants show prolonged mitotic phase. This may relate to an effect on histone H4. Histone deacetylation facilitates the formation of condensed chromatin which occurs after DNA duplication and prior to the M stage of cell cycle. SIRT2 deacetylates lysine 16 of histone H4 (H4Lys16) (Buck, Gallo et al. 2004). Levels of acetylated H4Lys16 decrease during the G2/M cell cycle transition coinciding with SIRT2 localisation to chromatin(Vaquero, Scher et al. 2006).

At different time points during mitosis, SIRT2 is associated with the centrosome, the mitotic spindle and the midbody. SIRT2 regulates the APC/C complex which triggers the transition from metaphase to anaphase(Kim, Vassilopoulos et al. 2011) and this group found SIRT2 null cells showed more mitotic abnormalities than normal cells. Overexpression of wildtype, or catalytically inactive SIRT2

increases multinucleation, possibly due to a failure to complete the mitotic separation(North and Verdin 2007).

1.14.4 Transcription regulator

SIRT2 deacetylates eukaryotic translation initiation factor 5A (eIF5A), in association with HDAC6 causing acetylated eIF5A to accumulate in the nucleus (Ishfaq, Maeta et al. 2012). Histone 3 is acetylated on lysine 56 as a necessary part of chromatin formation, localises to sites of repair after DNA damage and H3K56Ac levels are increased in many types of cancer. H3K56Ac (histone 3 acetylated on lysine 56) is deacetylated by SIRT1 and SIRT2(Das, Lucia et al. 2009) SIRT3 (Vempati, Jayani et al. 2010) and SIRT6(Michishita, McCord et al. 2009).

Autoacetylation of p300 occurs readily in the presence of Acetyl CoA but it is largely deacetylated in human cells. SIRT2 – uniquely among the human sirtuins – deacetylates p300 and allows it to bind with VP16 and GAL4 in an essential step in formation of the preinitiation complex for mammalian genetic transcription(Black, Mosley et al. 2008). Conversely, p300 interacts with SIRT2, which is acetylated and thus downregulated, completing a negative feedback loop (Han, Jin et al. 2008).

SIRT2 deacetylates and downregulates p53 (Jin, Kim et al. 2008).

1.15 SIRT3

1.15.1 Gene location, subcellular localisation and tissue expression

The SIRT3 gene is located on chromosome 11p15.5, close to the telomeric DNA(Frye 1999; Onyango, Celic et al. 2002). It consists of 7 exons which encode a protein of 399 amino acids.

SIRT3 mRNA is widely expressed in human adult and foetal tissues; expression is specifically reported in colon tissue samples in the adult(Frye 1999). It is localised to the mitochondrial subfraction and has definite NAD-dependent deacetylase activity (Onyango, Celic et al. 2002). Mitochondrial import of SIRT3

requires the presence of an N-terminal sequence, and the 43.6kDa SIRT3 is cleaved within the mitochondrial matrix to form a protein of 28kDa weight, and thereby activated(Schwer, North et al. 2002). In a range of 12 adult tissues, SIRT3 expression showed remarkably little variation, in contrast to other sirtuins.

Initial reports commented that endogenous expression levels were very low in U2OS and HeLa cells and confined to the mitochondria(Cooper and Spelbrink 2008). However, using highly specific antibodies to the N-terminus and C-terminus, Scher et al placed SIRT3 in the nucleus, with translocation of a processed form to the mitochondria; export increased on exposure to UV irradiation or DNA damaging agent(Scher, Vaquero et al. 2007). The short form was only detected in mitochondria but both were evident in the nucleus, suggesting processing occurs in the nucleus to direct mitochondrial transfer.

SIRT3 translocates to the nucleus on over expression with SIRT5 in COS7 (monkey fibroblast-derived) cells(Nakamura, Ogura et al. 2008). This may be the mechanism by which it achieves access to genes for transcription. A putative nuclear targeting signal was identified; mutations within this sequence prevent nuclear localisation of both SIRT3 and a proportion of the SIRT5 signal.

Currently it is generally accepted that the majority of SIRT3 is mitochondrial, but nuclear and cytoplasmic functions are discussed below.

Mitochondrial (short form) SIRT3 is protected, whereas the long form has a short half-life of 30 minutes. Further truncation of SIRT3, likely occurring within the mitochondrial matrix, increases its activity against certain substrates and may be relevant in a wider context in determining substrate specificity ranges, and rate of catalysis(Schlicker, Gertz et al. 2008). Both N- and C-terminal processing thus have demonstrable effects.

1.15.2 Association with lifespan

SIRT3 is the only one of the human sirtuins shown to have an effect on human longevity(Rose, Dato et al. 2003). A single nucleotide polymorphism increases longevity in males only. This team also identified a VNTR (variable nucleotide

tandem repeat) sequence in intron 5 of the SIRT3 gene which is commoner in the oldest (over 80 years) section of a cohort of Italian males(Bellizzi, Rose et al. 2005). Again, there was no difference in allele distribution by age in females.

1.15.3 Association with transcription

SIRT3 represses transcription by deacetylation of H4K16 and H3K9(Scher, Vaquero et al. 2007). Targets include PTK2, a cell adhesion molecule which reduces mobility, GAL3ST1, a sulphotransferase involved in metabolism of many drugs and OTOG, a glycoprotein expressed in the inner ear which may have functions in cell adherence.

Further actions of SIRT3 are described in later sections but in summary, it is linked to cancer progression as it regulates production of ROS which may be tumorigenic, and it is a key regulator of oxidative phosphorylation and the balance between this and the less-efficient energy production of glycolysis.

However, SIRT3 null mice do not display an obvious phenotype when young and have normal ATP levels in liver, heart and kidney. Since several other sirtuins are also implicated in pathways that confer resistance to oxidative stress, energy metabolism and cell fate and longevity, it raises the question how the sirtuins interact in the context of different or several stressors. At present we have evidence of many interesting relationships, and the full picture of interactions of any sirtuin will be very complicated but it may be the interplay between them that is essential to an understanding of how they integrate pathways such as metabolism and cell division.

1.16 SIRT4

1.16.1 Gene location, subcellular localisation and tissue expression

The SIRT4 gene is located on chromosome 12q(Frye 1999) and resembles prokaryotic sirtuin sequences. It has ADP-ribosylation activity on histones(Haigis, Mostoslavsky et al. 2006). Deacetylation activity has not been demonstrated so far.

SIRT4 is widely expressed in human adult and foetal tissues but colonic expression levels are comparatively low(Frye 1999). SIRT4 localises to the mitochondria on immunofluorescence and Western blot of subcellular fractions and in human and mouse cells (Michishita, Park et al. 2005; Haigis, Mostoslavsky et al. 2006). An N-terminal signal sequence is cleaved during mitochondrial import(Ahuja, Schwer et al. 2007) and SIRT4 is localised as a soluble protein within the mitochondrial matrix.

1.17 SIRT5

1.17.1 Gene location, subcellular localisation and tissue expression

SIRT5 is located as a single gene on chromosome 6 and is transcribed in two isoforms(Mahlknecht, Ho et al. 2006). The longer full-length form encodes a 310 amino acid protein and the shorter version includes a variation in the last exon, a 299 protein(Matsushita, Yonashiro et al. 2011). SIRT5 sequence is more similar to prokaryotic sirtuins than to yeast Sir2 protein(Frye 1999).

SIRT5 is widely expressed in adult and foetal human tissues, particularly in testis, skeletal muscle, kidney and heart; colonic mRNA expression is at comparatively high levels(Frye 1999). SIRT5 was first reported to be localised to mitochondria in human fibroblasts and HeLa cells(Michishita, Park et al. 2005).

COS7 cells are fibroblast-like cells of simian origin. Using these cells, SIRT5 was more precisely located in the mitochondrial intermembrane space(Nakamura, Ogura et al. 2008). Interestingly, when coexpressed at supraendogenous levels with SIRT3, SIRT5 signal remained in the mitochondria whereas SIRT3 signal was seen in the nucleus. When a putative nuclear targeting signal of SIRT3 was mutated (SIRT3nu), SIRT3nu and a proportion of SIRT5 signal were distributed within the cytoplasm.

These results may indicate that SIRT5 interferes with SIRT3nu localisation to mitochondria, or it could be that SIRT3 and SIRT5 bind together for nuclear targeting. Transport of SIRT5 into the mitochondrial matrix has been confirmed in mouse hepatocytes and also showed the existence of a typical N-terminal

sequence cleaved on import into the mitochondrial matrix(Nakagawa, Lomb et al. 2009).

Mouse cerebellar neurons were reported to show localisation of SIRT5 to nucleus and cytoplasm as well as mitochondria, although in a subpopulation of these cells only mitochondrial localisation was found. Cells in this subpopulation were more susceptible to apoptosis(Pfister, Ma et al. 2008).

The two different isoforms of SIRT5 identified in human cells were investigated further and it was found that isoform 2 has a shorter C-terminal sequence(Matsushita, Yonashiro et al. 2011). When overexpressed in HEK293, COS7 and HeLa cells, isoform 1 localised to the nucleus and cytoplasm, while isoform 2 localised to the mitochondria. Unlike the mouse cerebellar neurons reported previously, none of these cell lines showed exclusive mitochondrial localisation. These investigators concluded that the longer C-terminal sequence was necessary for nuclear and cytoplasmic localisation. SIRT5 isoform 2 appears to be quickly degraded in cytoplasm by the proteasome; the stability of the longer form seems important, but there may also be yet unidentified targeting interactions for which the C-terminus is required.

1.17.2 Enzyme activity and susceptibility to NAD

SIRT5 acetylation activity has been shown, but is less efficient than that of SIRT1-3(Du, Zhou et al. 2011). By contrast, Du et al showed that its active site can also accommodate malonyl and succinyl groups, and removal of these groups occurs with much greater (29-100 times) efficiency than the deacetylation reaction. Carbamoyl phosphate synthetase 1 (CPS1), which is known to undergo deacetylation by SIRT5, also undergoes desuccinylation, and succinylation levels of specific lysine residues are much higher in Sirt5 knockout mice.

Malonyl-CoA and succinyl-CoA are present in HeLa cells, and function in fashions similar to acetyl-CoA as donors in protein modification(Peng, Lu et al. 2011). SIRT5 is the only HDAC to demonstrate significant lysine demalonylation or desuccinylation, and that knockdown of SIRT5 changes global lysine demalonylation and desuccinylation, but has little effect on global deacetylation.

SIRT5 desuccinylation activity is sensitive to NAD, due to the presence of a specific arginine amino acid near the active site, which may also underlie the desuccinylase activity of SIRT5.(Fischer, Gertz et al. 2012).

Thus although SIRT5 can and does deacetylate in the classic sirtuin fashion, it appears that this may not be its most important mode of action. SIRT5 seems to be one that expands the sirtuin sphere of influence into other protein modification pathways.

1.18 SIRT6

1.18.1 Gene location and subcellular localisation

SIRT6 is localised to chromosome 19p13.3(Frye 2000; Mahlknecht, Ho et al. 2006). In the first report of its subcellular localisation, in human fibroblasts, SIRT6 was found in the nucleus, associated with heterochromatin but excluded from the nucleolus (Michishita, Park et al. 2005). Similar nuclear expression of SIRT6 protein was reported in fibrosarcoma cells, but light cytoplasmic staining was described in embryonic stem cells(Mostoslavsky, Chua et al. 2006).

A more recent investigation into the subcellular localisation of SIRT6 in HeLa cells reported exclusive nuclear location of the protein, but also showed that it was enriched in the nucleolus with variability in the degree among different cells(Ardestani and Liang 2012). S phase cells show little nucleolar staining while there is a significant increase in G1 phase. SIRT6 levels increase in mitosis but it is spread throughout the cytoplasm and associates in part with α-tubulin and mitotic spindles.

Cytoplasmic staining in breast cancer cells (Khongkow, Olmos et al. 2013) is explored further below.

1.18.2 Enzymatic activity

In initial experiments, only lysine deacetylase activity against histone H3 in 2 locations was noted (Kawahara, Michishita et al. 2009; Michishita, McCord et al. 2009). SIRT6 has ADP-ribosylation activity, and was reported to act on itself(Liszt, Ford et al. 2005). Other targets include PARP1, further discussed in

connection with the role of SIRT6 in DNA repair (Mao, Hine et al. 2011). Unlike other sirtuins, SIRT6 can bind NAD in the absence of an acetylated substrate(Pan, Feldman et al. 2011). ADP-ribose and NAD appear to induce different conformational changes and SIRT6 does not accommodate NADH in its binding site, in contrast to other sirtuins. Thus its regulation may be also be different.

1.18.3 Role in regulation of base excision repair (BER)

Investigating the effect of SIRT6 deficiency, it was noted that SIRT6-null mouse embryonic fibroblasts (MEFs) and embryonic stem cells (ESCs) showed increased sensitivity to IR but normal UV sensitivity(Mostoslavsky, Chua et al. 2006). There were higher numbers of metaphase chromosomes with abnormalities such as fragmentation, detached centromeres, gaps and translocations. BER rather than cell cycle checks or double strand break (DSB) repair mechanisms was confirmed as the underlying defective process.

Consistent with these findings, SIRT6 null cells showed increased sensitivity to alkylating agents and damage due to ROS, which tend to cause single strand breaks repaired by BER.

However, surprisingly, SIRT6 did not localise with the base excision repair machinery, although over expression of components of the BER restored wildtype function after treatment with alkylating agents. The specific action of SIRT6 was not clearly demonstrable.

1.18.4 Role in double strand DNA break repair

SIRT6 mobilises the DNA-protein kinase subunit of the DSB repair complex and stabilises it at chromatin adjacent to DSB (McCord, Michishita et al. 2009). SIRT6 also interacts with CtIP, a protein crucial for the DNA resection step in DSB repair(Kaidi, Weinert et al. 2010)

SIRT6 ADP-ribosylates PARP1, which autoactivates and, by ADP-ribosylation, recruits repair proteins to DSB (Mao, Hine et al. 2011) in human fibroblasts. SIRT6 significantly increases the rate of NHEJ in experimental conditions of

oxidative stress. PARP1 is involved in BER and in DSB repair by the DNA-dependent and alternative (DNA-independent) pathways.

Homologous recombination, the more precise mechanism for repair of DSB, decreases with onset of replicative senescence in human fibroblasts, in association with decreasing levels of SIRT6, and other proteins(Mao, Tian et al. 2012). This phenotype can be rescued by increasing SIRT6 expression and it is due to SIRT6 mono-ADP-ribosylation of PARP1, rather than interaction with CtIP.

1.18.5 Role in telomere maintenance

Given the reduced lifespan in Sirt6 null mice, Michishita et al investigated the effects of SIRT6 depletion in cells(Michishita, McCord et al. 2008). SIRT6 knockdown human fibroblasts (S6KD cells) showed more end-to-end chromosomal fusions, and more telomere dysfunction-induced foci, although mean telomere length was not shorter. hTERT expression reversed signs of premature replicative senescence seen in S6KD cells. SIRT6 localised to telomeric chromatin in S-phase, and S-phase was longer in S6KD cells.

This team also indentified lysine 9 of histone 3 (H3K9) as the first confirmed deacetylation target for SIRT6, and showed that its activity is responsible for maintaining low levels of H3K9Ac at telomeres in S-phase. WRN is. The telomere fusion sites in S6KD cells had weak telomeric signals, implying shortening of telomeres prior to fusion, rather than lack of telomeric capping proteins. The conclusion drawn was that SIRT6 and WRN (a DNA helicase, responsible for the Werner syndrome, a profound progeria) interact to stabilise telomeres and prevent premature replicative senescence. Absence of this interaction leaves telomeres vulnerable to and underlies the premature ageing phenotype seen in Sirt6 null mice.

SIRT6 has also been shown to deacetylate H3K56Ac, which localises to telomeric chromatin and increases dramatically at G2/M phase(McCord, Michishita et al. 2009).

The telomere position effect refers to the epigenetic silencing of genes close to telomeres. Maintenance of this state requires SIRT6, likely through its action on

H3K9Ac, which promotes the closed chromatin structure which inhibits transcription(Tennen, Bua et al. 2011).

1.19 SIRT7

1.19.1 Gene location, subcellular localisation and tissue expression

SIRT7 is a single copy gene, localised to chromosome 17q25.3, and translated as a single isoform of 400 amino acids (Voelter-Mahlknecht, Letzel et al. 2006). Like SIRT4 and SIRT6, it lacks conserved amino acids thought necessary for deacetylation(Tanny and Moazed 2001), but deacetylation of p53 by murine Sirt7 has been reported (Vakhrusheva, Smolka et al. 2008).

SIRT7 was predicted to localise mainly to the nucleus, but cytoplasmic and mitochondrial targeting signals were also noted(Dryden, Nahhas et al. 2003). In the original immunohistochemistry report using normal human fibroblast cells overexpressing SIRT7, expression in the nucleus and most prominently in the nucleolus was observed(Michishita, Park et al. 2005).

The first finding of cytoplasmic SIRT7 staining was reported in human fibroblasts and colonic epithelium, as well as the expected nucleolar staining (Kiran, Chatterjee et al. 2013). Heavy cytoplasmic staining, but no nuclear staining was observed in normal human colon epithelial cells on immunohistochemistry. Two isoforms of SIRT7 were identified, with molecular weights of 45 and 47.5 kDa respectively. The former localises to the nucleus in fibroblasts and epithelial cells, and the latter to the cytoplasm, in fibroblasts only. The same paper reported that fibroblasts which developed replicative senescence lost the nucleolar staining gradually with increasing passage; this change was not seen in cells with prematurely induced senescence. There was an inverse and reciprocal relationship between levels of the 45kDa and 47.5kDa isoforms in young and senescent fibroblasts (less 45kDa SIRT7 was identified in older cells).

Following from this, epithelial cells in G2/M arrest, or held at end of mitosis had an increased proportion of 47.5kDa SIRT7 and a decreased proportion of 45kDa SIRT7. The opposite effect was obtained by serum starvation, which maintained cells in G1/S phase. Treatment of the cells with phosphatase did not change the

proportion of 47.5kDa SIRT7 detected, implying that differences between isoforms is due to posttranslational processing other than phosphorylation alone.

1.19.2 Interaction with RNAPol1

SIRT7 in mouse tissue was associated with coding and promoter sequences of rDNA(Ford, Voit et al. 2006). 25% of SIRT7 complexes co-precipitated with RNAPol1. rRNA synthesis decreased in cells transfected with siRNA to SIRT7 and mutations affecting enzymatic activity did not interfere with nucleolar localisation but did reduce rRNA synthesis and association of Pol1 with rDNA. Selective inhibition of rRNA synthesis released SIRT7 from the nucleolus. The specific substrate of SIRT7 in its association with Pol1 and rDNA was not identified, and its enzymatic action was likewise not discerned. Neither deacetylation nor ADP-ribosylation of the histone substrates tested was observed.

Grob et al found that activation of SIRT7 is required for the resumption of rDNA transcription in late stages of mitosis(Grob, Roussel et al. 2009). Proteomic analysis of SIRT7 interactions has shown significant interactions with RNAPolII as well as Pol1, and with B-WICH, a chromatin remodelling complex(Tsai, Greco et al. 2012). SIRT7 deacetylates H3K18Ac (acetylated lysine18 on histone H3), and reduces transcription of a specific set of genes, primarily involved with RNA transcription and processing, and with transcription of ribosomal proteins(Barber, Michishita-Kioi et al. 2012). This implies that SIRT7 may have a wider impact on rDNA transcription as well as the specific associations demonstrated so far, and further results are awaited.

The following paragraphs describe common areas of influence of the sirtuins of particular relevance to this thesis, with description of areas of sirtuin interrelationships.

1.20 Sirtuins and control of metabolism

1.20.1 SIRT2: maintenance of substrate availability

In adipocytes, by overexpression and in low insulin and low glucose states, SIRT2 inhibits differentiation (Jing, Gesta et al. 2007) and promotes binding of FOXO1 to PPARγ, reducing transcription of its targets(Wang and Tong 2009).

Through another pathway inhibited by HIF1α, SIRT2 deacetylates PGC1α (Krishnan, Danzer et al. 2012). This in turn increases transcription of PPARγ and expression of beta-oxidation and mitochondrial gene targets, hindering the development of a state of obesity. SIRT2 deacetylates phosphoenolpyruvate carboxykinase (PEPCK1), the rate-limiting enzyme in gluconeogenesis, stabilising it(Jiang, Wang et al. 2011). SIRT2 therefore seems to maintain availability of substrate in conditions of scarcity, but its actions on PPARγ appear to depend on which upstream pathway is activated.

1.20.2 SIRT4: Switch to fatty acid catabolism from glucose and protein substrates

SIRT4 levels in the liver were reduced when the animals were fed a high fatty acid diet, and elevated in response to a high fructose diet(Chen, Fang et al. 2010).

SIRT4 knockdown increases expression of mitochondrial and fatty acid oxidation genes in mouse hepatocytes, and increases rates of fatty acid oxidation(Nasrin, Wu et al. 2010). This was associated with increased SIRT1 protein and mRNA levels. In vivo SIRT4 knockdown in mice resulted in upregulation of SIRT3 in hepatocytes and slight decrease in blood glucose. Investigations on mouse myotubes confirmed SIRT4 has a role in negative regulation of mitochondrial oxidative metabolism in these cells. These results suggest that SIRT4 may be instrumental in regulating SIRT1 and to a lesser extent SIRT3 expression. Nuclear localisation has not so far been reported for SIRT4 and it will be interesting to find out how it achieves these effects.

Investigation of peripheral blood cells in diabetic and normal human subjects showed decreased levels of SIRT4 (and SIRT1) in the diabetic cases(Song, Xu et al. 2011). This team also noted negative correlation between fasting glucose and markers of high-risk lipids (triglycerides and lipoprotein A), and SIRT4.

1.20.2.1 Effect on insulin signalling

SIRT4 ADP-ribosylates and inhibits GDH in mouse and human cell lines at endogenous levels. Leucine stimulates GDH so that amino acids are used as fuel for the tricarboxylic acid cycle (TCA) and directly and via this alternative production of energy, stimulates insulin secretion. (This process of amino acid-stimulated insulin secretion is abbreviated to AASIS). SIRT4 null mice have higher insulin secretion in response to glucose and show more AASIS. SIRT4 may coordinate pancreatic and hepatic metabolism, decreasing AASIS in the pancreas and reducing the use of amino acids for gluconeogenesis in the liver.

Experiments on human cell lines identified further targets for SIRT4 ADP-ribosylation involved in insulin signalling. Insulin-degrading enzyme is a metalloproteinase which regulates amyloid-β and insulin levels. ADP/ATP translocases 2 and 3 exchange ATP generated by respiration in the mitochondrial matrix for cytosolic ADP(Ahuja, Schwer et al. 2007).

1.20.3 SIRT1: sensitivity to insulin

SIRT1 influence on metabolic processes varies according to the tissue under investigation, and is tailored to integrate tissue-specific functions into a whole-body homeostatic network(Imai and Yoshino 2013). Most of the available data comes from mouse models. Liver-specific knockout mice are more prone to hepatic steatosis on a high fat diet, with impaired PPARγ function, reduced fatty acid β-oxidation and more endoplasmic reticulum stress(Purushotham, Schug et al. 2009). SIRT1 deficiency in another model interfered with mTOR/AKT signalling, resulting in chronic hyperglycaemia, oxidative stress and insulin resistance, on a normal diet(Wang, Kim et al. 2011).

Similarly, SIRT1 seems to improve white adipose tissue sensitivity to insulin, through action on PPARγ (Qiang, Wang et al. 2012). In skeletal muscle of mice under caloric restriction, SIRT1 deactivates STAT3, altering availability of subunits of PI3K and effecting a more efficient insulin-stimulated response in PI3K(Schenk, McCurdy et al. 2011).

1.20.4 SIRT1 and SIRT3: substrate availability in starvation

Acetyl CoA synthase (AceCS) 1 and 2 convert free acetate into a usable metabolite in mammals; endogenous sources are important for use of acetate as a substrate in starvation. AceCS1 is located in the cytoplasm, AceCS2 in the mitochondrial matrix(Fujino, Kondo et al. 2001). Acetate metabolism is impaired as humans age(Skutches, Holroyde et al. 1979). SIRT1 deacetylates and activates AceCS1, improving use of acetate in lipid synthesis. SIRT3 has very similar activity but lack of function in whole cell analyses probably results from the subcellular confinement of SIRT3 to the mitochondrial matrix. Deacetylation by SIRT3 reactivates AceCS2 with 5 times greater effect than SIRT1(Hallows, Lee et al. 2006; Schwer, Bunkenborg et al. 2006). Increased AceCS2 activity increases CO2 output whereas increased AceCS1 activity has no such effect. This suggests that different pools of AcetylCoA exist with different sirtuin regulatory mechanisms and possibly different functions in increasing energy storage by lipid synthesis, versus increasing energy output by increased respiration.

1.20.5 SIRT3: shift in balance from glycolysis to enhanced oxidative phosphorylation

SIRT3 germline knockout mice showed increased susceptibility to oxidative stress and significant metabolic dysfunction. Surprisingly, mice with SIRT3 knockdown in a tissue-specific manner do not show any particular phenotypic variation although mitochondrial protein acetylation is increased(Fernandez-Marcos, Jeninga et al. 2012).

AceCS2 expression increases in ketogenesis. It is proposed that in fasting conditions fatty acids are released by adipose tissue, oxidised to acetate and ketone bodies in the liver and available for muscle after conversion to acetyl-CoA by AceCS2. Gene expression was studied in the context of hypoenergetic dieting in obese women (Capel, Viguerie et al. 2008). Subjects on a moderate fat/low carbohydrate hypoenergetic diet showed increased expression of SIRT3, consistent with a regulatory effect increasing fatty acid oxidation.

Within mitochondria, SIRT3 is localised to the mitochondrial matrix(Schwer, North et al. 2002). Its deacetylation activity is deemed to be important but given that the levels of NAD are generally high in this compartment it is not clear whether this is a rate-controlling factor or may serve merely to keep SIRT3

activity rate high. It is also possible that it is the NAD:NADH ratio which is more important for enzyme function than the absolute level of NAD.

Experiments with Sirt3 null mice show that SIRT3 promotes amino acid catabolism in low intake situations by activating ornithine transcarbamoylase and increased urea cycle throughput, along with alterations in β-oxidation of fatty acids(Hallows, Yu et al. 2011).

SIRT3 also regulates ATP levels by deacetylation of subunits of the electron transport chain Complex 1 in mice(Ahn, Kim et al. 2008). In human HeLa cells, both the long and short isoforms of SIRT3 interacted with Complex 1.

SIRT3 interacts with subunits of electron transport chain complexes 2 and 5, succinate dehydrogenase A (SDHA), and ATP synthase subunit O-mitochondrial (ATP5O) respectively. SIRT3 deacetylates SDHA, and its activity is reduced in Sirt3 null mice(Finley, Haas et al. 2011).

Palmitate (a free fatty acid) stimulates ROS accumulation and production of cytokine MCP1 in tubular cells of a mouse model of proteinuric kidney disease(Koyama, Kume et al. 2011). SIRT3 is decreased in these cells and MCP1 inversely correlated; SIRT3 overexpression reduced ROS levels and increased antioxidant gene expression, linking it to lipotoxic ROS-mediated inflammation. This may be of relevance in human metabolic syndrome and associated disease.

ATP levels are thought to be important in initiation of cell death by apoptosis or necrosis. Thus there are at least 2 pathways by which SIRT3 could influence cell fate. SIRT3 deacetylates and activates glutamate dehydrogenase (GDH) which is deactivated by ADP-ribosylation by SIRT4(Schlicker, Gertz et al. 2008), and also deacetylates and activates isocitrate dehydrogenase, a key regulator of the citric acid cycle, both of which increase ATP production.

SIRT3 is central to a shift in balance from glycolysis to enhanced oxidative phosphorylation in one cell line through deacetylation of cyclophilin D. (Shulga, Wilson-Smith et al. 2010) Cyclophilin D is necessary to maintain hexokinase II binding to voltage-dependent anion channels which preserve mitochondrial

integrity. This may be of relevance in transformed cells, but will depend on SIRT3 levels. As shown below, SIRT3 levels in cancer cells are variable.

SIRT3 interacts with hypoxia-inducible factor 1α (HIF1α). Finley et al (Finley, Carracedo et al. 2011) showed that increased reactive oxygen species, such as may result from SIRT3 knockdown, decrease the activation of prolyl hydroxylases which by adding hydroxyl groups to HIF1α enable binding of von Hippel-Lindau protein and targeting of HIF1α to degradation by the proteasome. Thus decreased SIRT3 results in increased levels of HIF1α, with increased expression of genes controlling glycolysis. This may offer one mechanistic explanation for the Warburg effect, a tendency of cancer cells to use glycolysis to generate energy even in the presence of adequate oxygenation. They also linked this knockdown with increased glycolysis in breast cancer cells and with increased cancer cell proliferation in these lines. SIRT3 is shown to be reduced in several breast cancers and in other epithelial cancers.

1.20.6 SIRT5: Regulation of throughput in protein catabolism

In vitro and physiological investigation showed that Sirt5 deacetylates carbamoyl phosphate synthetase 1 (CPS1) in murine hepatocytes(Nakagawa, Lomb et al. 2009). CPS1 catalyses the combining of ammonia and bicarbonate to form carbamoyl phosphate, the first step in the urea cycle. Sirt5 knockout mice show lower levels of CPS1 activity and build-up of ammonia when subjected to caloric restriction, as they are unable to adequately handle the ammonia produced when amino acids are used for gluconeogenesis. Since neither Sirt5 nor CPS1 protein levels increases in CR, it appears that increased NAD levels activate SIRT5 which in turn activates CPS1. Experimental results show that intramitochondrial NAD levels and CPS1 activity both increase in caloric restriction in wildtype but not Sirt5 KO mice.

Proteomic analysis of mouse cells showed over 700 proteins with 2,500 lysine succinylation sites(Park, Chen et al. 2013). Global levels of lysine succinylation increase in response to fasting, and show a higher baseline in Sirt5-deficient mice. A subset of specific proteins showing higher levels of lysine succinylation in Sirt5-deficient mice included histones and ribosomal proteins, and over half of proteins involved in fatty acid metabolism and the TCA. A significant proportion

of these function outside of mitochondria, and immunofluorescence confirmed localisation of SIRT5 in the cytosol. Proof of principle experiments showed that two of the proteins identified (pyruvate dehydrogenase complex and succinate dehydrogenase (in electro transport chain Complex II) showed more lysine succinylation and increased activity in SIRT5 knockdown in HeLa cells, and SIrt5 null mouse cells respectively.

SIRT5 appears to have an important part to play in regulation of gluconeogenesis using amino acid sources.

1.20.7 SIRT1 and SIRT6: Balancing lipid metabolism

Both rat and human cell lines showed increased SIRT6 protein expression when grown in nutrient-deprived serum(Kanfi, Shalman et al. 2008). Similar effects were seen in rat and mouse tissues after caloric restriction regimes. This increase was achieved by stabilisation of SIRT6 protein which is usually degraded by the proteasome pathway.

SIRT6 in mouse cells is positively regulated by SIRT1 in complex with FOXO3a and NRF1 through their combined effect on the SIRT6 gene promoter, in conditions of glucose starvation(Kim, Xiao et al. 2010). SIRT6 deficiency causes reduced expression of genes involved in glycolysis and synthetic lipid metabolism, by deacetylation of H3K9 at their promoters and hepatic knockdown of SIRT6 causes development of fatty liver in mice.

HIF1α regulates a metabolic switch to glycolysis under nutrient-deprived conditions. HIF1α levels are higher in SIRT6 deficient cells as a result of both increased protein synthesis and stability. All of the genes upregulated in SIRT6 deficient cells are targets of HIF1α, and SIRT6 binding to the promoters is contingent on interaction between SIRT6 and HIF1α. This may relate to H3K9 deacetylation of SIRT6 in the region of glycolytic gene promoters or it may be a separate regulatory mechanism.

Sterol-regulatory element-binding proteins (SREBPs) regulate lipid metabolism. MicroRNAs within their introns have a negative effect on expression of targets including fatty acid β-oxidation genes as well as SIRT6, and a subunit of PKA and

IRS2 (Horton, Goldstein et al. 2002; Horton, Goldstein et al. 2002). SIRT6, AMPK and IRS2 tend to counteract the lipogenic effects of SREBP, placing SIRT6 within a complex regulatory framework for lipid metabolism.

SIRT1 decreases acetylation of PGC-1α, to activate it and thus increase expression of target genes to promote gluconeogenesis(Rodgers and Puigserver 2007). SIRT6 indirectly inhibits PGC-1α in U2OS and HEK293A cells, (Dominy, Lee et al. 2012) through the action of SIRT6 on GCN5, which acetylates PGC1α. Overexpression of Sirt6 in mouse primary hepatocytes did reduce expression of gluconeogenic genes as expected. Indeed, knockdown of SIrt6 in whole animal studies increased hepatic gluconeogenesis and glucose output and expression of key gluconeogenic genes.

An important link between inflammation and metabolism has recently been reported, identifying a change from the acute stage of inflammation, dependent on glycolysis to a later adaptive phase where fatty acid oxidation is essential(Liu, Vachharajani et al. 2012). The adaptive response phase requires NAMPT synthesis of NAD. TLR4-stimulation increases expression of SIRT1 and SIRT6, and SIRT1 activation also stimulates SIRT6 expression. SIRT1 and SIRT6 knockdown experiments show that SIRT6 inhibits glycolysis and SIRT1 activates the fatty oxidation pathways. Clinical relevance of these results comes from assessment of leukocytes from human patients with sepsis, in late stage as assessed by TNFα expression. The human leukocytes exhibited the expected increase in fatty acid metabolism with reduction in rates of glycolysis.

1.21 Sirtuins and control of inflammation

Several of the sirtuins act through effects on transcription of subunits of NFκB. This process is influenced by TNFα with evidence of a feedback loop involving SIRT6.

1.21.1 SIRT1 and inflammatory responses

SIRT1 deacetylates the RelA/p65 subunit of NFκB, inhibiting its transcriptional activity(Yeung, Hoberg et al. 2004). This leaves cells more vulnerable to TNFα

mediated apoptosis (in contrast to the anti-apoptotic effects of SIRT1 deacetylation of p53).

1.21.2 SIRT2 and inflammatory responses

SIRT2 deacetylates p65, which is a component of NFκB(Rothgiesser, Erener et al. 2010). Hyperacetylation of p65 in SIRT2 null cells after TNFα stimulation results in expression of a subset of NFκB target genes.

1.21.3 SIRT6: Associations with inflammation

SIRT6 inhibits transcription of NFκB target genes, by deacetylation of H3K9Ac in the region of NFκB promoters (Kawahara, Michishita et al. 2009). It is recruited to these promoters by interaction with the RelA subunit of NFκB. This interaction is inducible by TNFα, which also promotes nuclear transfer of NFκB for transcription of target genes, but interestingly, decreases the transcription of NFκB target genes by destabilising the interaction between RelA and the promoter. Both SIRT6 and RelA homozygous deficiency are lethal in mice, but SIRT6-null, RelA heterozygotes survive much longer, suggesting that some of the lethal effect of the SIRT6 deficiency is mediated by excess NFκB action.

TNFα protein synthesis in inflammatory leukocytes is regulated by SIRT6 by posttranslational modification(Van Gool, Galli et al. 2009)

SIRT6 and TGFβ expression was investigated in idiopathic pulmonary fibrosis (IPF) (Minagawa, Araya et al. 2011), where TGFβ1 induced senescence in IPF cells, via p21 activity, independently of p53. SIRT6 overexpression reduces p21 activity by labelling p21 mRNA for degradation and can prevent senescence. SIRT6 overexpression also reduces IL1β expression; since IL1β also functions as a paracrine signal, inducing myoepithelial differentiation in neighbouring cells, it is conceivable that SIRT6 and IL1β affect surrounding epithelial cells in lung and other tissues. This may be relevant to SIRT6 interactions in cancer as well as other inflammatory pathologies.

Specific examination of the role of SIRT6 in inflammation was performed using HUVECs exposed to LPS(Lappas 2012). SIRT6 knockdown was associated with

increased levels of IL1β, IL6, IL8, MMP2, MMP9, PAI1, ICAM1 and VEFG and FGF2. It would be useful to know if these observations persist in colonic epithelial cells.

1.22 Sirtuins and control of cell fate

Inflammatory responses instigated by TNFα are interlinked with the effect of sirtuins on cell fate.

1.22.1 SIRT2 and cell death

TNFα instigates cell death by necrosis, acting through SIRT2 and the RIP3-RIP1 complex which initiates the cell death process. Hearts of SIRT2 null mice show some protection from ischaemia reperfusion injury and TNFα is ineffective in the face of SIRT2 inhibition(Narayan, Lee et al. 2012) which suggests therapeutic potential for modulation of SIRT2 function.

SIRT2 is upregulated by a GSK3 inhibitor and induced apoptosis via a caspase-independent pathway(Pizarro, Folch et al. 2009). Decreased activity of SIRT2 can result in necrosis and apoptosis by caspase-3-dependent pathways(He, Nie et al. 2012). p53 deficient chicken cells were treated to prevent transcription of functional SIRT1 and SIRT2(Matsushita, Takami et al. 2005). SIRT2 deficient cells were more sensitive to pro-apoptotic stimuli, implying regulation of cell death pathways in p53-independent manner.

SIRT2 binds to FOXO3a, increasing its DNA binding, and elevating transcription of MnSOD and Bim. Bim is a propapoptotic factor, thus SIRT2 is part of the pathway which promotes cell death in cells under severe stress. Increased levels of MnSOD tend to reduce levels of ROS, normalising the oxidative stress.

SIRT2 depletion enhances anoxia-reoxygenation tolerance(Lynn, McLeod et al. 2008) and this effect is achieved through interaction with another 14-3-3 isoform, 14-3-3ζ, which interacts with Bad, a member of the Bcl-2 gene family which is involved in initiating apoptosis.

1.22.2 SIRT3: Association with cell cycle and cell death

After Bcl-2 knockdown in human colorectal carcinoma HCT116 cells, apoptosis increases. Silencing of SIRT3 has no effect on cell fate, but knockdown of both Bcl-2 and SIRT3 reduces the rate of apoptosis to that of wildtype cells(Allison and Milner 2007). In human epithelial non-carcinoma cell line ARPE19, Bcl-2 knockdown induced cell cycle arrest at G1 phase, rather than apoptosis. SIRT3 silencing alone had no effect on cell growth but dual silencing prevented the cell cycle arrest seen with knockdown of Bcl-2 alone.

JNK2 also inhibits apoptosis, in a p53-independent manner, in a pathway which requires JNK1 activity(Allison and Milner 2007). Again, after JNK2 knockdown, apoptosis increases. Silencing of SIRT3 has no effect on cell fate, but knockdown of both JNK2 and SIRT3 rescues cells from apoptosis. SIRT3 expression is increased by JNK1.

NAMPT catalyses the rate-limiting step in NAD recycling. Knockdown experiments showed that SIRT3 and SIRT4 are necessary for the protective effect of NAMPT, on treatment with methylmethane sulphonate, which causes cell death by hyperactivation of PARP(Yang, Yang et al. 2007).

1.22.3 SIRT4: Association with cell death

Opening of the mitochondrial permeability transition pore (PTP) in a sustained manner is a decisive event in irreversible cell injury. Verma et al showed that SIRT4 suppression prevents PTP opening in response to calcium and to calcium-independent mechanisms(Verma, Shulga et al. 2013). SIRT4 decreases mitochondrial capacity to store calcium, through its inhibition of GDH but the downstream mechanics of GDH action are not entirely clear. SIRT4 downregulation inhibited TNF-induced cell death, which is mediated via PTP, and this process also depended on GDH function.

The ability of the mitochondria to absorb calcium may be protective when calcium homeostasis is perturbed. SIRT4 expression may therefore be controlled as part of the cell's defence in cases of cell damage and loss of control of calcium containment.

1.23 Sirtuins and cancer

There is a wide-ranging literature on sirtuin mRNA expression in a variety of cancers, and a smaller body of work describing sirtuin protein levels as assessed by immunohistochemistry. Both expression levels and associations with disease progression or survival are very variable. Data on associations with colorectal cancer are highlighted.

1.23.1 SIRT1 and cancer

Context appears to be important in assessing SIRT1 function in cancer. Two studies commented on changes in staining intensity in colorectal cancer tissue alone; in one, loss of SIRT1 staining associated with progression along the adenoma-carcinoma sequence (Jang, Min et al. 2012). In another, 30% of tumours showed lower SIRT1 expression, 25% of Stage 1-3 tumours showed overexpression and SIRT1 expression tended to be lower in more advanced tumours(Kabra, Li et al. 2009).

Studies have reported SIRT1 overexpression in pancreatic(Zhao, Cui et al. 2011), prostate(Wang, Hasan et al. 2011), liver (Chen, Zhang et al. 2011), ovarian (Jang, Kim et al. 2009) and gastric cancers (Cha, Noh et al. 2009), and melanoma(Nihal, Ndiaye et al. 2011). Wang et al reported reduced SIRT1 expression in 44 breast cancer samples in comparison to 25 unmatched normal control samples(Wang, Sengupta et al. 2008).

It appears that SIRT1 cannot be simply characterised as a tumour suppressor or as an oncogene. It may contribute to defence against malignant transformation, but once this has taken place, its functions in protecting the cell from apoptosis may be subverted in the interest of cancer cell immortality.

1.23.2 SIRT2 and cancer

No description of SIRT2 expression at the mRNA or protein level in colorectal cancer was indentified. SIRT2 is part of a three-gene biomarker panel that indicates worse outcome in oesophageal adenocarcinoma(Ong, Shapiro et al. 2013).

SIRT2 protein expression is associated with high PSA level, old age, high Gleason score and clinical progression and reduced survival time in a cohort of 110 cases

of prostate cancer(Hou, Chen et al. 2012). This analysis did not distinguish between nuclear and cytoplasmic staining for SIRT2.

Samples from a cohort of glioblastoma and diffuse astrocytoma cases showed that SIRT2 cytoplasmic staining was generally reduced in malignant versus normal specimens(Imaoka, Hiratsuka et al. 2012). The opposite was true of nuclear staining, which was higher in tumour specimens. Percentage of positively stained nuclei was calculated for each sample but cytoplasmic staining was categorised only absent or present. Lower percentage (<60%) of positively stained nuclei correlated with longer survival.

SIRT2-null mice developed increased numbers of tumours and these were gender-specific (mammary gland in females and various liver and GI cancers in males)(Kim, Vassilopoulos et al. 2011). This same group also found reduced protein expression in 36 human breast cancer specimens, and 18 metastatic breast cancer specimens compared with adjacent normal tissue. Of 264 hepatocellular carcinoma samples, protein levels were increased in 10, unchanged in 125 samples relative to normal unmatched liver tissue, and were reduced in 129 cases.

SIRT2 and HDAC6 are associated with increased cellular migratory and invasive potential in bladder cancer models(Zuo, Wu et al. 2012). This effect is likely to be achieved through action on tubulin and the cytoskeleton.

1.23.3 SIRT3 and cancer

No studies analysing at the mRNA or protein level in colorectal cancer were located but SIRT3 is implicated in apoptosis in colorectal cancer cell lines as an effector in both the Bcl-2 and JNK2 mediated pathways (Allison and Milner 2007). In this context its effects are contingent on the background nutritional and stress status of the cell.

In a human tumour microarray, immunohistochemical staining for SIRT3 was reduced in breast tissue with respect to normal tissue. It also correlated with pathological grade of tumour. It was reduced in a variety of other human tumours (head and neck squamous cell cancer, glioblastoma multiforme,

hepatocellular and clear cell renal cancer, testicular and prostate cancers). By contrast, SIRT3 overexpression is reported in oral squamous cell cancer(Alhazzazi, Kamarajan et al. 2011) and lymph node-positive breast cancer (Ashraf, Zino et al. 2006).

Following on from data linking SIRT3 to metabolic control were investigations linking SIRT3 and its regulation of mitochondrial ROS levels to cancer. SIRT3 null mice embryonic fibroblasts (MEFs) were shown to have increased levels of ROS and specifically mitochondrial ROS (Kim, Patel et al. 2010). This was associated with chromosomal instability with increased numbers of chromosomes after a modest dose of radiation. SIRT3 knockout mouse liver tissue and MEFs showed decreased mitochondrial DNA integrity over time. The MEFs did not tend to form tumours of themselves but when exposed to Myc or Ras overexpression became immortalised. In contrast, wild-type cells required expression of both Myc and Ras for immortalisation.

SIRT3 knockout MEFs showed more aneuploidy with overexpression of either or both of these genes. These cells also showed anchorage independence growth capacity, by their ability to grow in soft agar, and tumorigenic potential when transplanted into nude mice. Oxidative phosphorylation was also affected by removal of SIRT3 activity, with effects as previously predicted on Complex I and also on Complex III of the electron transport chain. These characteristics were not shown by wild-type SIRT3 cells with overexpression of either of Myc or Ras. These characteristics were also reduced by overexpression of MnSOD, suggesting that loss of SIRT3 activation of MnSOD underlies the permissive aspect of the SIRT3 knockout tumorigenic phenotype. There was an-age-associated variation in this response, suggesting that loss of SIRT3 is associated with loss of MnSOD primarily in older mice.

SIRT3 knockout mice tend to develop tumours more often than wild-type mice. These tumours tended to be of a well-differentiated type, expressing both oestrogen and progesterone receptors. This parallels the type of tumours found in older women. Murine SIRT3-null tumours also showed evidence of increased nitrosative and oxidative damage, postulated to occur as a function of age.

SIRT3 in human bladder cancer cell line associates with p53 in the mitochondria to prevent p53-induced growth arrest(Li, Banck et al. 2010).

1.23.4 SIRT6: Associations with cancer

SIRT6 gene expression is downregulated in a proportion of human colorectal and pancreatic cancers(Sebastian, Zwaans et al. 2012). SIRT6 nuclear protein expression is reduced in colorectal and pancreatic cancers and low SIRT6 is associated with node positive colorectal cancers and systemic inflammation, a poor prognostic factor for colorectal cancer. There was no specific mention of SIRT6 cytoplasmic staining. APC mutant mice which tend to develop multiple colonic polyps show more high grade and invasive neoplasms when the SIRT6 mutation is added.

SIRT6 expression using immunohistochemistry in breast cancer reported cytoplasmic staining as well as nuclear staining for the first time (Khongkow, Olmos et al. 2013). In 118 cases, high nuclear SIRT6 staining was associated with worse overall survival, and the converse was true for cytoplasmic staining. There was a trend towards worse disease-specific survival with higher nuclear SIRT6 staining.

In mice, deletion of c-Jun increases DEN-induced liver tumorigenesis with evidence of increased apoptosis (Min, Ji et al. 2012). In early cancers there was increased SIRT6 binding to the survivin promoter, and reduced acetylation of H3K9 at the same promoter. Dysregulation of several NFκB genes, and decreased binding of p65/NFκB to the survivin promoter was also found, in keeping with previously reported effects of SIRT6 on NFκB genes(Kawahara, Michishita et al. 2009). c-Fos increases SIRT6 transcription and is itself inhibited by c-Jun. c-Fos levels are higher after DEN treatment in c-Jun deficient liver cells.

c-Fos and SIRT6 levels were high in 11 healthy human liver samples, with low c-Jun and survivin; the pattern was reversed in 12 of 73 dysplastic liver nodules which are recognised as precursor lesions to hepatocellular carcinoma (HCC).

SIRT6 activity in relation to cancer was investigated in more general terms in mice: SIRT6 deficiency promoted tumour development and conferred a growth

advantage on transformed and non-transformed MEFs, which also exhibited increased glycolysis(Sebastian, Zwaans et al. 2012). Transformed SIRT6-deficient cells did not activate any of the downstream effectors of oncogenic activation but still maintained increased glycolysis. Notably, inhibition of the rate-limiting enzyme in glycolysis, PDK, inhibited anchorage-independent growth, reversed glucose-addiction (apoptosis induced by absence of glucose in growth medium) and reduced tumour formation in whole animals.

1.23.5 SIRT7: Associations with cancer

There was no data located on SIRT7 expression in colorectal cancer, but SIRT7 gene expression was much higher in thyroid cancer (papillary, follicular and anaplastic) cell lines and tissue samples than in cell lines derived from normal thyroid tissue, samples of normal thyroid tissue or samples from benign adenomas(de Nigris, Cerutti et al. 2002). SIRT7 expression is higher in breast cancer tissue than in normal tissue, and is higher in node-positive breast cancers(Ashraf, Zino et al. 2006).

SIRT7 expression was increased in a cohort of human hepatocellular carcinoma samples(Kim, Noh et al. 2013). Further investigation by this group suggested the existence of a regulatory pathway involving p53, which promoted microRNA inhibition of SIRT7. SIRT7 inhibits p21 expression and cell-cycle arrest in G1/S phase. Several cancer cells with higher levels of SIRT7 carried mutations in p53.

In 39 cases of head and neck squamous cell carcinoma, SIRT7 gene expression was reduced in tumour tissue relative to normal tissue(Lai, Lin et al. 2013). Protein immunohistochemistry showed staining in nuclei and was lower in tumour tissue, but not quantitatively analysed. Expression was progressively reduced with advancing cancer stage, but there was no relationship with survival.

There are widespread associations between the sirtuins and metabolic regulation, cell cycle control, and cancer, but there is a lack of information on protein expression in colorectal epithelium and in colorectal tumour tissue. Analysis of protein expression and associations with markers of

clinicopathological prognosis in colorectal cancer was therefore felt to be of interest.

1.24 Telomeres

Discovered (Blackburn and Gall 1978) soon after the seminal publication describing the Hayflick limit, telomeres were postulated to be the replicometer which counted cell divisions and prevented cell division past the predetermined limit. A neat corollary from this was the idea that critically short telomeres were the signal for sidetracking a cell into quiescence(Harley 1991).

1.24.1 Structure of telomeres

Telomeres are nucleoprotein complexes at the end of chromosomes. The DNA component in humans consists of the repeated hexamer TTAGGG; similar sequences in other species attest to evolutionary conservation of this motif. Telomeric DNA forms a buffer beyond coding sequences, necessary because the mode of action of the DNA polymerase requires an RNA template to begin elongation of the copied strand. This template is subsequently degraded and so a variable length of terminal DNA is lost with each cell division – the 'end-replication problem' which produces telomere shortening identified by Olovnikov(Olovnikov 1973).

The two ends of the DNA strands are bent back and hidden within an ordered loop structure(Griffith, Comeau et al. 1999). This protects the ends of DNA strands from damage and prevents exposure and recognition of the chromosome ends as double stranded DNA breaks. This conformation is achieved through the action of some of the shelterin proteins (de Lange 2005).

1.24.1.1 Shelterin complex

Six proteins form the shelterin complex which together with the looped DNA configuration hide and protect the ends of the DNA strands at the end of chromosomes. Several other proteins, notably those involved in DNA damage response like the MRN complex, and PARP2, associate with shelterin. They are not felt to be integral components of the complex, as the shelterin proteins are defined as those which are localised at telomeres only and remain there

throughout the cell cycle, and whose known functions are confined to telomeres. TRF1, TRF2 and POT1 bind the telomeric hexamer directly, and were discovered first from their predicted affinity for this sequence(Zhong, Shiue et al. 1992; Broccoli, Smogorzewska et al. 1997; Baumann and Cech 2001).

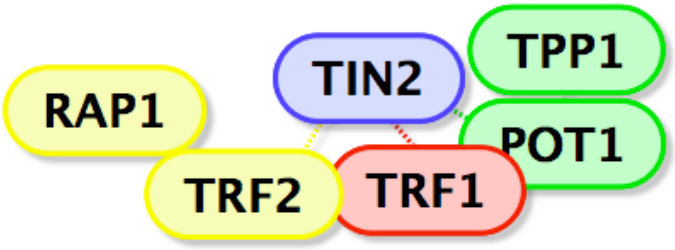

Figure 1.2 Schematic diagram of components of the shelterin complex.

TPP1 binds to POT1. TIN2 is of central important to the complex as it binds the heterodimer of TPP1/POT1 to both TRF1 and TRF2 and binds TRF1 and TRF2 together as well. RAP1 binds to TRF2.

The schematic above gives a basic representation of these interactions between the shelterin proteins but in vivo, multiple shelterin complexes bind at stretches of telomeric DNA and can adopt different conformations, varying also if other DNA-associated proteins are involved. This dynamic system responds to prevailing intracellular conditions. Alteration of the shelterin configuration, particularly with recruitment of DNA repair proteins to other sites of DNA damage can expose telomeric DNA to damage. This may be one of the means by which accelerated telomere shortening occurs.

1.24.2 Telomerase

Shelterin prevents degradation and shortening of telomeres by physically sequestering them, but it also controls the length of telomeres to a certain degree by regulating telomerase activity(Loayza and De Lange 2003). Telomerase can synthesise telomeric DNA and thus elongate telomeres. It is composed of a reverse transcriptase, encoded by the TERT (TElomerase Reverse Transcriptase) gene, and a template: TERC (TEmplate RNA Component).

Telomerase activity and TERT expression reduce soon after birth, but are maintained in cells which need to maintain telomere length. Telomerase is generally inactive in differentiated cells, but remains active at a low level in stem cells to support the necessary number of cell divisions required in the lifespan of an organism(Gunes and Rudolph 2013). Stem cell exhaustion with telomere shortening despite these mechanisms is one means by which ageing in a particular organ or compartment may be explained(Hiyama and Hiyama 2007). Mutations in the TERC or TERT genes have been shown to underlie dyskeratosis congenita and aplastic anaemia; the pathology in both diseases is explained by a failure of the haematopoietic stem cell compartment(Vulliamy, Marrone et al. 2002; Westin, Chavez et al. 2007).

1.24.3 Associations with telomere shortening

In keeping with the effect of repeated cell division, it is estimated that 6-11% of PBL telomere shortening is attributable to age(Weischer, Nordestgaard et al. 2013). Several other factors have been associated with an increased rate of telomere shortening, which may eventually overtake the physiological rate of replacement, if telomerase is activated, or attenuate telomere length to a problematic degree.

1.24.3.1 Sex

Telomere length in leukocytes, and umbilical artery endothelium is equivalent in male and female neonates(Okuda, Bardeguez et al. 2002). Reports of longer leukocyte telomere length in adult females have been suggested to offer some explanation for the longevity gender gap(Nawrot, Staessen et al. 2004). Males had a higher rate of telomere attrition in a cohort aged 0-100 years of age(Mayer, Bruderlein et al. 2006). Oestrogen activates telomerase in MCF-7 cells and if this effect was widespread in vivo could attenuate stem cell telomere shortening(Kyo, Takakura et al. 1999).

The most recent meta-analysis of this interaction has concluded that robust data on the gender-attributable variability of telomere length decrease with age is not available(Muezzinler, Zaineddin et al. 2013).

1.24.3.2 Oxidative stress

Oestrogen reduces oxidative stress(Aviv 2002) and this may be the explanation for longer leukocyte telomere length in females. Oxidative stress (the production of ROS in excess to what can be safely managed by the cell) is particularly damaging to telomeres because of their guanine-rich sequences(Oikawa and Kawanishi 1999) and repair of single-stranded DNA lesions is less efficient in telomeric regions(Petersen, Saretzki et al. 1998). A large study in a Scottish population identified 5 oxidative stress genes with robust associations with markers of functional bio-age, and linked 2 of these with effects on telomere length(Starr, Shiels et al. 2008). Oxidative stress has been mechanistically linked to accelerated telomere erosion by association with polymorphisms in uncoupling protein 2 (UCP2) in patients with diabetes(Salpea, Talmud et al. 2010). UCP2 is involved in mitochondrial production of ROS.

1.24.3.3 Smoking

Particulate matter air pollution is associated with increasing oxidative stress, telomere erosion and cardiovascular disease(Grahame and Schlesinger 2012). Smoking is a classic paradigm of such pollution and has also been linked with telomere shortening(Babizhayev, Savel'yeva et al. 2011). Smoking also has potent links with cancer, and the interaction between telomere length and cancer will be discussed further in subsequent sections.

1.24.3.4 Inflammation

One of the effects of cigarette smoking is to induce a chronic inflammatory state. Inflammation also increases oxidative stress. Short telomeres in leukocytes have been correlated with IL-6 levels in patients with COPD, independent of smoking history(Savale, Chaouat et al. 2009). Uncontrolled viraemia was associated with shorter leukocyte telomere length in HIV-infected children(Cote, Soudeyns et al. 2012). Of course, leukocytes are part of the inflammatory response so that short telomeres in these cells may reflect immune activation and increased numbers of cell divisions, as much as any ageing effect of an inflammatory response.

In patients with rheumatoid arthritis, muscle cell telomere length was shorter than in a control group with osteoarthritis(Duijnisveld, Bigot et al. 2011). Colorectal mucosal cell telomeres are shorter in patients with longstanding UC

with respect to non-colitics (O'Sullivan, Bronner et al. 2002). Short telomeres associated with ulcerative colitis in colorectal mucosa are thought to be a part of the tumorigenic process in IBD-related colorectal cancer(Risques, Lai et al. 2011).

1.24.4 Effects of critically short telomeres: senescence

There are thus a variety of pluripotential factors potentially causative for telomere attrition. The effects of telomere shortening are comparably important.

1.24.4.1 Senescence

After a relatively constant number of population doublings, cell in culture enter a state of replicative senescence, characterised by a cessation of division and s senescence-associated secretory phenotype. This state is associated with significant telomere shortening relative to the starting length (Harley, Futcher et al. 1990). Cells with shorter telomeres undergo less population doublings than cells which begin with longer telomeres before entering replicative senescence (Allsopp, Vaziri et al. 1992) so there seems to be a threshold telomere length to trigger the change(Hayflick 2000).

1.24.4.2 Aneuploidy

Senescent cells are initially cleared by the innate immune system(Hornsby 2010), but with time, their numbers accumulate. In this scenario, ongoing telomere shortening is possible, particularly in situations where cell-cycle checkpoints are malfunctioning, to cellular ‘crisis’. Once telomere shortening has proceeded to such a degree, the secondary loop structure of the telomere can no longer be maintained. The shelterin complex may not be able to bind and the telomere ends are exposed and stimulate the same reaction as any double stranded DNA break(von Zglinicki, Saretzki et al. 2005). These ‘breaks’ are ‘repaired’ by non-homologous end-joining, creating dicentric chromosomes, which break at a different point during the next cell division(Murnane 2012). This cycle can repeat, with consequent recurrent translocation of large swathes of DNA, causing measurable genomic instability and aneuploidy.

1.24.5 Telomere length and cancer

The sequence described in the preceding paragraph is very reminiscent of one at least of the Hallmarks of Cancer delineated in an earlier section. There is an extensive literature on telomere length in cancer because of the associations with aberrant cell division and also because telomere length is an attractive candidate Biomarker of Ageing. It is worth noting that much of this literature deals with leukocyte telomere length and therefore associations between cancer and ageing on a whole-organism level, but there is also an extensive examination of telomere length in specific ageing tissues, and association with cancer in that particular compartment.

The telomere hypothesis of carcinogenesis at a cellular level involves shortened telomeres, crisis and genomic instability. Activation of telomerase is also a necessity in the transformed phenotype to maintain replicative potential(Kim, Piatyszek et al. 1994). In colorectal epithelium, there is an established adenoma-carcinoma sequence(Plentz, Wiemann et al. 2003) which has leant itself to investigation of the telomere hypothesis of carcinogenesis, and several reports have demonstrated shorter telomere lengths in tumour than in adjacent non-malignant mucosa, evidence of ageing at a tissue level(Valls Bautista, Pinol Felis et al. 2009; Maxwell, McGlynn et al. 2011; Valls, Pinol et al. 2011).

The process of reactivation of telomerase and its place in malignant transformation was assessed initially by correlation in normal mucosa, adenomas and carcinomas in colorectal and gastric mucosa, with some studies showing detectable telomerase at the adenoma stage (Maruyama, Hanai et al. 1997)The variation in telomere length through the sequence from crisis to immortalisation was analysed in detail in spontaneously immortalising Nijmegen breakage syndrome T-cells (Degerman, Siwicki et al. 2010). These cells showed attrition of telomeres to the point of crisis, when telomere lengths were very short. Cells escaped from crisis by aberrant DNA damage response and repression of senescence controlling genes, but telomere length remained very short. Subsequently, TERT expression increased in association with c-Myc expression, telomerase activity and elongation of telomeres to a certain degree. This implies that telomerase expression may be quite a late event in the process of immortalisation.

Reactivation of telomerase is seen in 90% of cancers(Shay and Bacchetti 1997). A further small proportion use a process called Alternative Lengthening of Telomeres (ALT) for elongation(Henson, Neumann et al. 2002) which happens by a process of homologous recombination between sister chromatids(Bechter, Zou et al. 2004) Since most data indicates that tumour tissue telomere length is shorter than telomere length in adjacent normal tissue, it seems that telomere length is maintained above the level of crisis, but not particularly higher, assuming that the cells analysed are at a range of points along the spectrum described by Degerman et al. However, there are also sporadic but recurrent reports of tumour tissue with extremely long telomeres. Ten-fold variability in telomere length in not uncommon in cells employing ALT and there is some evidence to suggest that it carries a poorer prognosis(Valls, Pinol et al. 2011).

1.24.6 Telomere Length as a Biomarker of Ageing (and Disease)

Telomere length presents as an attractive candidate Biomarker of Ageing. The ‘miles on the clock’ hypothesis proposes that telomere length functions as a measure of the biological age of an individual because telomere length integrates attrition due to the increasing chronological age through the end-replication problem, and accelerated attrition from ambient stressors. This is analogous to a car which will deteriorate gradually as time passes even if it merely sits idle, but will wear out faster if it accrues more miles on the clock.

Telomere length may integrate several influences which separately, additively or synergistically produce telomere shortening. It is thought by many that leukocyte telomere length provides an accessible surrogate measure of ageing in different tissues, with integration of ageing influences from many tissues to give an overall summary of the ‘agedness of the organism’.

In a recent review of telomere length and susceptibility to disease, 47 case-control studies were reviewed and all but 2 showed an association between short telomeres and the disease in question, or an adverse change of variable or lifestyle characteristic(Bojesen 2013). 26 studies tabulated in this same paper assessed the association between short telomere length and mortality in a wide

range of diseases. 10 of the studies investigated populations over 70 years of age; 9 of these were included in the 11 studies which showed no association.

Two of the studies showing no association were in cancer patients with age ranges 30-90 years (breast and renal cancer)(Svenson, Nordfjall et al. 2008; Svenson, Ljungberg et al. 2009). In the breast cancer patients, there was a significant association between short telomere length and survival in patients under 50 years, but this was not the case for patients aged over 50. The study in older people which did show an association between short telomeres and mortality was performed in twins aged 73-94(Kimura, Hjelmborg et al. 2008). It showed that shorter length of the shortest 25% of telomeres predicted the mortality of that twin better than mean telomere length.

These results attest to a relationship between short leukocyte telomeres and adverse outcomes. It also appears that in an unselected population, shorter telomere lengths in leukocytes are associated with increased mortality. It may show evidence of 'premature ageing' but reliable measurements which take account of inter-individual variation have limited its usefulness so far. In older people (age above 70 may be a reasonable definition of 'older', on the basis of the studies reviewed) the relationship between leukocyte telomere length and age appears not to be maintained (Martin-Ruiz, Gussekloo et al. 2005; Martin-Ruiz, Jagger et al. 2011).

The 26 studies reviewed above used leukocyte telomere length in all but 1 case. The generalisability of the association between leukocyte telomere length and mortality to specific other tissues is unproven(von Zglinicki and Martin-Ruiz 2005). The assumption of telomere length correlation among all tissues within the body is repeatedly made, but there is a large degree of inter-individual variation in telomere length. The volume of data available may not be large enough to adjust for the variation noted. This inaccuracy may not affect the usefulness of leukocyte telomere length as a marker of whole-organism bioage. It may introduce confusion in interpretation of the totality of literature on telomere length and ageing as applied to specific diseases. The literature on telomere length in different tissues is reviewed in a subsequent chapter.

1.24.7 Measurement of telomere length

The method of telomere length measurement is important. Interpretation of telomere length results in the literature has been complicated by variability in terminology and methods of measurement of telomere length(Shiels 2010). Methods of measurement of telomere length vary in applicability to different tissues, scaleability and the level of specificity of measurement. Measurement of telomere length by any means in specific tissues is limited to some extent by the ability to purify cells of that tissue.

1.24.8 Terminal Restriction Fragment Length

The classical method of measurement of telomere length uses restriction endonucleases which cleave DNA in the subtelomeric region. Frequent cutting restriction enzymes digest non-telomeric DNA and the remaining fragments are run on an agarose gel. Depending on the endonuclease used, a variable amount of subtelomeric DNA is included. Southern blot is performed with radio-labelled probes specific to the telomere sequence. Densitometric analysis of the autoradiograph produced allows measurement of the average length of these telomere terminal restriction fragments.

This method allows quantitative measurement of telomere length in kilobase pairs. It is a relatively cumbersome process which takes time and a substantial amount of tissue is required for the extraction of sufficient DNA.

The average telomere length measured is calculated from all nucleated cells in the sample, so it provides data at a cell population level. It is hypothesised that the location of the restriction enzyme cleavage site in the subtelomeric regions may vary between individuals and depending on the endonuclease used. All of these are factors in the variability in the data obtained.

1.24.1 FISH/Q-FISH and Flow Cytometry

Quantitative fluorescence in-situ hybridization (Q-FISH)) is used to measure telomere length in metaphase spreads. Probes emit fluorescent signal when bound to the DNA under investigation. The signal is proportional to the number of hybridized probes and thus to the telomere length. Results correlate well with the Southern blot technique but this technique is even more specialised. It also requires the use of dividing cells from which to make metaphase spreads. Cell

division is less likely in senescent tissues, so that they may be underrepresented in telomere measurement.

Unlike TRFL measurement, since telomere lengths are measured in individual cells and even on individual chromosomes, a specific analysis of telomere length for defined populations of cells is possible.

In situ hybridization was combined with flow cytometry to increase the throughput possibilities and generalisability of the technique. Cells can be fixed, and permeabilised to allow nuclear insertion of telomere specific fluorescent probes. The process also requires heating to DNA denaturation temperatures (>80ºC) to allow binding of the probe. While allowing for tissue fixation, the need for these high temperatures may limit the applicability of immunohistochemical identification of cells as specific epitopes may also be denatured.

1.24.1 Measurement of Relative Telomere Length

Relative telomere length analysis of DNA using real-time polymerase chain reaction as described by Cawthon is in routine use within our laboratory (Cawthon 2002). This process uses DNA polymerase in a polymerase chain reaction to amplify specific sequences of DNA present in small quantities in a sample to more easily measurable amounts, from which the initial concentration, or relative concentration, of the sequence under investigation can be estimated.

Repeated cycles of DNA denaturation, annealing of specific primers and synthesis of the complementary strand gradually increase the number of copies of the sequence of interest. The primers limit the length of the DNA sequence repeatedly synthesized. The DNA polymerase used is from a thermophilic bacterium, which is stable at high temperatures. It is not destroyed by the high temperatures necessary to denature the DNA strands between cycles of amplification. Sufficient amounts of the 4 bases must be added so they do not limit the reaction rate.

1.24.1.1 Real-time PCR

Real-time PCR is a modification of the PCR process to give a quantitative assessment of how many copies have been formed as cycles progress. SYBR Green fluoresces only when bound to double-stranded DNA (dsDNA), and the fluorescence level is therefore proportional to the amount of dsDNA which has been formed during the synthesis part of each cycle.

Maximum fluorescence is measured during each cycle and plotted against cycle number. The cycle number at which fluorescence rises above the background level is known as the crossing point (Cp). Given that, in theory the amount of DNA present doubles with each cycle, it is possible to estimate the relative concentration of the original DNA sample by backwards extrapolation using the number of cycles needed to achieve the crossing point. In fact, the efficiency of the reaction (the actual increase in DNA copy numbers) is rarely as high as 2 but is an important marker of the reliability of the experiment. (See below in reference to the single copy reference gene.)

Measurement of telomere length in terms of the number of telomeric repeats is made difficult by the repetitive DNA sequence of which they are composed. This favours the formation of primer dimers, when the primers bind to each other instead of to the DNA. Cawthon developed two primers whose structures are sufficiently different to each other to prevent the formation of primer dimers but can bind to the telomeric DNA(Cawthon 2002).

1.24.1.2 Single copy reference gene

Telomere length varies between cell types, and between experimental subjects and it is this that we are trying to measure. Since it is formed of a hexameric repeat sequence, primers can bind at many points on its length and various lengths of product will be synthesized but the total amount of DNA copied will be proportional to the length of telomeric repeats present. A reference point is therefore needed. The reference used is a single copy gene which is present in all cells; 36B4, the gene coding for ribosomal protein, large P0 (also known as RPLP0) is the reference used in this method.

The measurement obtained is actually a ratio of telomere repeat copy number to single copy gene copy number for the experimental sample, relative to the

same ratio for an arbitrarily determined control. All experimental samples are normalized to this control, and so can be compared with each other in the normalized form. This normalized ratio is the T/S ratio. The efficiency of reaction for the telomere plate and the single copy gene plate must be comparable in order for the two to be compared.

This is the method of telomere measurement used in the experiments described in this thesis. Reproducibility of results and comparison between different investigators have been limited by variability in protocols used and reliability of the process. The protocol used in this laboratory is highly standardised and processes to assess and ensure reliability of results are described in the chapter on Experimental Methods.

Like the TRFL method, this measurement of relative telomere length by real-time PCR analyses telomere length in all nucleated cells within a sample. As such, the data obtained represents a mean telomere length for all cell types within the sample.

1.24.2 Limitations of telomere length measurement methods

This subject is explored in more detail on the chapter on telomere lengths in different tissues. Briefly, unless specific steps are taken to purify cell types within tissue samples, the telomere length measurement obtained will be an average from all nucleated cells in the sample. In colonic tissue for example, this may include stromal cells, or cells from neighbouring muscle layers. Within any one cell type, there may be a range of telomere lengths resulting from variable expression of telomerase in stem cells. Comparison of telomere length in individual cell types using the common denominator of TRFL might allow an assessment of the magnitude of this effect, but this data is so far not available. It is important that protocols specify tissue preparation steps to allow adequate understanding of the cell types that are likely to be included. This would aid assessment of the comparability of results within the field.

1.25 Clinicopathological factors investigated: rationale for selection

There are many clinical and pathological factors associated with outcome in colorectal cancer. The selection of certain specific factors for comparison with the experimental variables of telomere length, serum fetuin A and colorectal tumour sirtuin expression in this thesis is explained as follows.

1.25.1 Demographic factors

Assessment of relationship with chronological age is important as a baseline in the context of assessment of biological ageing. Sex has been associated with potential measures of biological ageing such as telomere length(Aviv, Shay et al. 2005). There is increasing evidence to implicate obesity in the pathogenesis of colorectal cancer(Giovannucci, Ascherio et al. 1995; Larsson, Rutegard et al. 2006; Pischon, Lahmann et al. 2006; Sinicrope, Foster et al. 2010). BMI was used as a surrogate marker of obesity.

1.25.2 Inflammatory markers

Serum C-reactive protein (CRP) is a non-specific marker of inflammation. Inflammation has been recently accepted as the newest hallmark of cancer(Colotta, Allavena et al. 2009). CRP, as a measure of the systemic inflammatory response, has been associated with a poorer prognosis for many types of cancer, including colorectal cancer (McMillan, Elahi et al. 2001).

Albumin is a negative acute-phase protein and hypoalbuminaemia is associated with poorer outcome from cancer(McMillan, Watson et al. 2001). As such, it has been incorporated into the modified Glasgow Prognostic Score, which has been validated as a prognostic tool in many types of cancer, including colorectal cancer(Forrest, McMillan et al. 2003; Elahi, McMillan et al. 2004; Hilmy, Bartlett et al. 2005; Al Murri, Bartlett et al. 2006; Crumley, McMillan et al. 2006; Glen, Jamieson et al. 2006; Al Murri, Wilson et al. 2007; McMillan, Crozier et al. 2007; Ramsey, Lamb et al. 2007).

1.25.3 Atypical inflammatory markers

Anaemia and high platelet count reflect inflammation which is often sub-clinical and chronic. Anaemia or rectal bleeding is often a presenting symptom of colorectal cancer. Low haemoglobin has recently been reported as an

independent risk factor for mortality from non-cardiac surgery(Leichtle, Mouawad et al. 2011; Musallam, Tamim et al. 2011). This may reflect an increasing biological age in anaemic patients, particularly since anaemia is also associated with chronic disease.

Thrombocytosis has been associated with poor prognostic features in colorectal cancer and with worse survival from the disease(Cravioto-Villanueva, Luna-Perez et al. 2012; Ishizuka, Nagata et al. 2012; Ishizuka, Nagata et al. 2012; Kawai, Kitayama et al. 2013).

Smoking induces a systemic inflammatory response, and has been associated with increased colorectal cancer risk(Paskett, Reeves et al. 2007; Liang, Chen et al. 2009; Kato, Boleij et al. 2013; Parajuli, Bjerkaas et al. 2013).

1.25.4 Comorbid conditions and antioxidant levels

Presence of other comorbid conditions may indicate increased biological age. Serum antioxidant levels may mitigate the effect of reactive oxygen species on telomere shortening. Data on total number of comorbid conditions and on serum antioxidant levels were available for the telomere analysis cohort. The Charlson comorbidity index has prognostic value in longitudinal studies. Data was available for the sirtuin immunohistochemistry cohort.

1.25.5 Factors of prognostic significance in colorectal cancer

In colorectal cancer, there are 2 widely recognised systems of staging. Dukes' staging system was described in the early 20th century(Dukes 1949) but has remained in use because of its simplicity and clinical relevance for prognosis. The criteria for the 4 stages in Dukes' classification are outlined below.

Dukes' Stage	Description
A	Tumour confined to the mucosa
B	Tumour spread beyond the mucosa but not metastasized to lymph nodes
C	Tumour spread to lymph nodes but not beyond the gut. This was later divided into C1 (affecting local lymph nodes) and C2 (affecting the apical lymph node).
D	Metastases in different organs

Table 1.2 Dukes' stages of colorectal cancer and defining criteria

The other widely used staging system is the TNM system which defines criteria relevant to tumour (T), nodal spread (N) and presence of distant metastases (M). Each component is relevant individually in certain circumstances and can be analysed separately. They can also be combined into an overall numerical American Joint Committee on Cancer (AJCC) staging. A comparison of staging systems in common use is presented in the table below.

AJCC stage	T	N	M	Dukes'
O	Tis	N0	M0	-
I	T1	N0	M0	A
	T2	N0	M0	A
IIA	T3	N0	M0	B
IIB	T4a	N0	M0	B
IIC	T4b	N0	M0	B
IIIA	T1-T2	N1/N1c	M0	C
	T1	N2a	M0	C
IIIB	T3-T4a	N1/N1c	M0	C
	T2-T3	N2a	M0	C
	T1-T2	N2b	M0	C
IIIC	T4a	N2a	M0	C
	T3-T4a	N2b	M0	C
	T4b	N1-N2	M0	C
IVA	Any T	Any N	M1a	D
IVB	Any T	Any N	M1b	D

Table 1.3 Comparison of AJCC staging with T, N, M components and Dukes' staging for colorectal cancer.

These systems largely define extent of spread of the cancer. Specific histological features of the resected colorectal tumour specimen are also relevant. Grade of tumour, assessed as degree of differentiation is a recognised prognostic factor, with poorly differentiated tumours generally being accepted as more aggressive with a worse prognosis. Several histological features of prognostic significance identified on examination of the resected specimen have been combined to form the Petersen Index(Petersen, Baxter et al. 2002). These are venous invasion, margin involvement, peritoneal involvement and tumour perforation. Tumour perforation carries a score of 2 in this model, while the other factors carry a score of 1. Low risk Petersen Index (score 0 or 1) is associated with significantly better survival in Dukes' B cancer.

Peritumoural inflammation is becoming recognised as a factor in better prognosis (Roxburgh, Salmond et al. 2009). Data on peritumoural inflammation was only available for the sirtuin immunohistochemistry cohort.

1.26 Statement of Research Aims

1.26.1 Statement of Aims: Prospectively collected matched tissue and blood cohort

To measure telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue in patients with colorectal cancer.

To measure fetuin A levels and markers of inflammation in serum from the same patients.

To evaluate any correlation between telomere lengths in matched samples from PBLs, normal colorectal tissue and colorectal tumour tissue.

To correlate levels of fetuin A and other serum inflammatory markers with telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue, in patients with colorectal cancer.

To correlate telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue of patients with colorectal cancer with clinicopathological markers of prognostic value in colorectal cancer.

To correlate levels of fetuin A with other markers of inflammation in serum, and with accepted clinicopathological markers of prognostic value in colorectal cancer.

1.26.2 Statement of Aims: TMA cohort

To stain a colorectal tissue microarray (TMA) containing normal and tumour tissue with antibodies to human sirtuins 2-7 and measure staining using the weighted Histoscore method.

To compare staining patterns in normal and colorectal tumour tissue.

To correlate staining for sirtuins 2-7 with cancer-related mortality and with clinicopathological markers of prognostic value, and serum markers of inflammation.

To compare protein expression levels to mRNA levels of the human sirtuins in normal and colorectal tumour tissue, and to compare protein levels in normal and tumour tissue.

To assess any interrelationships between protein expression levels of the human sirtuins 2-7 in normal colorectal tissue and in colorectal tumour tissue.

2 Materials and Methods

2.1 Patient Cohorts

Tissue used in the experiments described in this thesis came from two separate cohorts of patients. One cohort of patients contributed blood and colorectal normal and tumour tissue for the measurement of telomere length in matched tissues and for the measurement of serum fetuin A levels. Tissue from a second cohort of patients was used to construct the tissue microarray (TMA) for sirtuin immunohistochemistry (IHC). Patient inclusion in each cohort is further described below.

2.2 Ethical Approval

Ethical approval was obtained from Glasgow Royal Infirmary Research and Ethics Committee (REC ref number 08/S0704/22) for the measurement of telomere lengths and of serum fetuin A. A substantial amendment was completed to allow collection of blood samples from other hospitals in the same NHS Board, and expand the analysis of blood samples to measure RNA levels of sirtuins. This is not a focus of this thesis but is a logical extension of this project. Colorectal normal and tumour tissue specimens were obtained on application to the Glasgow Royal Infirmary Biobank.

2.3 Patient recruitment

2.3.1 Telomere length cohort

All patients diagnosed with colorectal cancer at Glasgow Royal Infirmary, Gartnavel General Hospital and the Western Infirmary, Glasgow from 10 July 2010 to 10 June 2011 were invited to participate in this study. 97 chose to do so and gave informed consent for the use of blood samples. Information on relevant clinicopathological parameters was gathered from the medical notes.

2.3.2 Sirtuin immunohistochemistry cohort

277 patients were diagnosed with operable colorectal cancer in the Greater Glasgow area between 1997 and 2007 and were considered for inclusion in this

cohort. These patients received standard (neo)adjuvant chemoradiotherapy according to local protocols at the time of diagnosis. Patients were excluded if tissue blocks were not available or contained insufficient tumour tissue for research purposes, as determined by the assessing consultant pathologist. The final cohort numbered 272 patients. Information on relevant clinicopathological parameters was obtained from a database prospectively maintained by MR James Park.

Clinical and pathological characteristics of the patient cohorts are summarised in the relevant chapters of this thesis with results of data analysis.

2.4 Collection and preparation of tissue samples for telomere length and fetuin A measurement.

2.4.1 Blood Sample Collection

Informed consent was gained from patients by EM prior to surgery and blood samples were taken in EDTA to allow DNA and RNA extraction, and in sodium heparin to allow analysis of antioxidants and vitamin levels. Within 1 hour, 1ml of blood in EDTA was mixed with 3ml of TRIzol to stabilize RNA and allow later analysis of RNA levels. All samples were transported back to the laboratory on ice. The details of further treatment protocols necessary to allow storage and later analysis of blood samples are described in the following sections.

2.4.2 Initial Preparation of Blood Samples

2.4.2.1 Preparation for RNA Analysis

The blood in TRIzol was vortexed thoroughly, divided in 4 aliquots and stored at -80°C.

2.4.2.2 Extraction of DNA from peripheral blood leukocytes

The first 30 samples were divided. Half of the sample was centrifuged at 1400g for 15mins, and the precipitant portion retained, with the aim of improving capture of leukocytes and thus better DNA yield. The remainder of the sample was processed as whole blood without centrifugation. DNA yield was not significantly improved by centrifugation and whole blood was used for DNA

extraction for the remaining 67 samples. DNA extraction was performed using the Maxwell® 16 Instrument (Promega) and Blood DNA Purification Kit cartridges, according to the manufacturer's instructions. DNA was stored in elution buffer at -20°C until analysed.

2.4.2.3 Processing of serum for Fetuin A ELISA

Samples were centrifuged at 1400g for 15mins to separate serum from red cells and buffy coat. Serum from the samples in EDTA was aliquoted off and stored at -20°C for ELISA.

2.4.2.4 Processing of serum for antioxidant and vitamin analysis

200ul of serum in sodium heparin was thoroughly mixed with an equal volume of 6% metaphosphoric acid and vortexed thoroughly to denature enzymes and stabilize Vitamin C content. The remaining serum was divided into 2 aliquots, ensuring no contamination with packed red cells. The interface serum and buffy coat was discarded. Packed red cells were divided into 2 aliquots and all 5 aliquots stored at -80°C until transfer to a partner laboratory at the Department of Biochemistry, Glasgow Royal Infirmary for analysis.

2.4.3 Colorectal Tissue Samples

Informed consent for tissue donation to the Glasgow Royal Infirmary Biobank was sought routinely by clinical staff at the time of preassessment, or at the time of consent for surgery for resection of colorectal cancer. Where this consent was available, the resected specimen was assessed by a consultant pathologist and, once sufficient tissue had been examined and retained as necessary for diagnostic purposes, surplus tissue was stored at -80°C in the Biobank. On successful application to the Biobank by EM, tissue was transferred to our laboratory on dry ice and stored at -80°C until analysed.

2.4.4 Extraction of DNA from Colorectal Normal and Tumour Tissue

DNA extraction was performed using the Maxwell® 16 Instrument (Promega) and Tissue DNA Purification Kit cartridges, according to the manufacturer's instructions. DNA was stored in elution buffer at -20°C until analysed.

2.5 Measurement of Telomere Length

Relative telomere length analysis of DNA using quantitative polymerase chain reaction as described by Cawthon (Cawthon 2002) was used in the following experiments, and it is further described below.

2.5.1 Plate Preparation for QPCR reaction

2.5.1.1 Plate Layout

96-well plates were used with layout as indicated in the table below. Two plates were run together, one with telomere-specific primers and the other with 36B4 primers. This minimized the variation between the paired plates as aliquots of the same samples were used for each plate. Primer sequences are detailed in Appendix 1. Each well was loaded with the relevant primers, DNA sample and constituents for the PCR reaction.

	Columns											
Rows	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	SD1	SD1	SD1	1	1	1	9	9	9	17	17	17
B	SD2	SD2	SD2	2	2	2	10	10	10	18	18	18
C	SD3	SD3	SD3	3	3	3	11	11	11	19	19	19
D	SD4	SD4	SD4	4	4	4	12	12	12	20	20	20
E	SD5	SD5	SD5	5	5	5	13	13	13	21	21	21
F	SD6	SD6	SD6	6	6	6	14	14	14	22	22	22
G	SC	SC	SC	7	7	7	15	15	15	23	23	23
H	NTC	NTC	NTC	8	8	8	16	16	16	24	24	24

Table 2.1 Layout of 96 well plate for RT-PCR measurement of telomere length. SD = standard dilution, SC = standard calibrator, ntc = no-template control. Numbers in wells indicate sample number; all standards and samples were analysed in triplicate.

2.5.1.2 Standard Curve and Control Samples

A standard curve was constructed for each plate. The control DNA sample was heated to 65°C for 10 minutes to resuspend the DNA, vortexed and the concentration was measured on a Nanodrop spectrophotometer. A starting solution with concentration 20ng/μl was made from the control DNA sample. This is standard dilution sample SD1. A serial dilution of the SD1 1:1 with nuclease-free water produced standard dilution samples SD2 to SD6 with decreasing concentrations from SD1 at 20ng/μl to SD6 at 0.625ng/μl. The standard dilutions were made in bulk and divided into aliquots of 70μl. These

were stored at 4°C. Before use, they were heated again to resuspend the DNA and vortexed before use, to ensure homogeneous aliquots were analysed.

Negative and positive controls were also used on each plate. The negative control contained only nuclease-free water (a no-template control – ntc). The positive control contained control DNA at a concentration midway along the standard curve. This was the standard calibrator (SC) and acted as the reference sample to which all experimental samples were normalized.

In initial experiments, a standard calibrator containing 10ng DNA/μl (50ng DNA total per well) was used, as was standard practice in this laboratory, but it was difficult to achieve a reliable standard curve. A standard calibrator containing 5ng DNA/μl (25ng DNA total per well) improved the reliability of the standard curve. The starting concentration of the control DNA was lower than in previous experiments, and therefore there was little dilution required to form the standard dilution solutions for this analysis. It may be that components of the elution buffer for DNA extraction were present in high enough concentrations to interfere with the PCR reaction in the more concentrated standard calibrator.

2.5.1.3 Experimental Sample Preparation

24 samples were run in triplicate on each 96-well plate. 35ng DNA is required as a solution of 7ng/μl per well, a volume of 5μl. Both telomere and 36B4 plates were prepared together. Thus 6 aliquots of each sample were needed to load 2 plates in triplicate. Samples were prepared to a total volume of 70μl (14 aliquots) to allow for a second run if the first was unsuccessful.

To make the 7ng/μl "telomere dilutions", DNA samples were heated to 65°C for 10 minutes to resuspend the DNA and vortexed; their concentration was measured on a Nanodrop spectrophotometer. The relevant volumes of DNA sample and nuclease-free water were calculated to produce the total volume of 70μl, with concentration 7ng/μl. These "telomere dilution" samples were stored at 4°C until used. If storage exceeded 24 hours, they were heated again to resuspend the DNA and vortexed before use, to ensure homogeneous aliquots were analysed.

2.5.1.4 Plate Preparation

SYBR Green Master Mix (Roche) and telomere and 36B4 primers (Invitrogen) were combined using PCR grade water to the manufacturer's instructions. Plates were loaded with 5μl standard or sample DNA per well, and then with 15μl Master Mix containing the appropriate primer per well, sealed, centrifuged and analysed on the Roche LightCycler LC480.

Telomere and 36B4 plate running conditions are described in Appendix 2.

The LightCycler plots the fluorescence output from bound primers against PCR cycles for each of the 96 wells in a plate. This produces an amplification curve for each well: an example is shown in Figure 1.1.

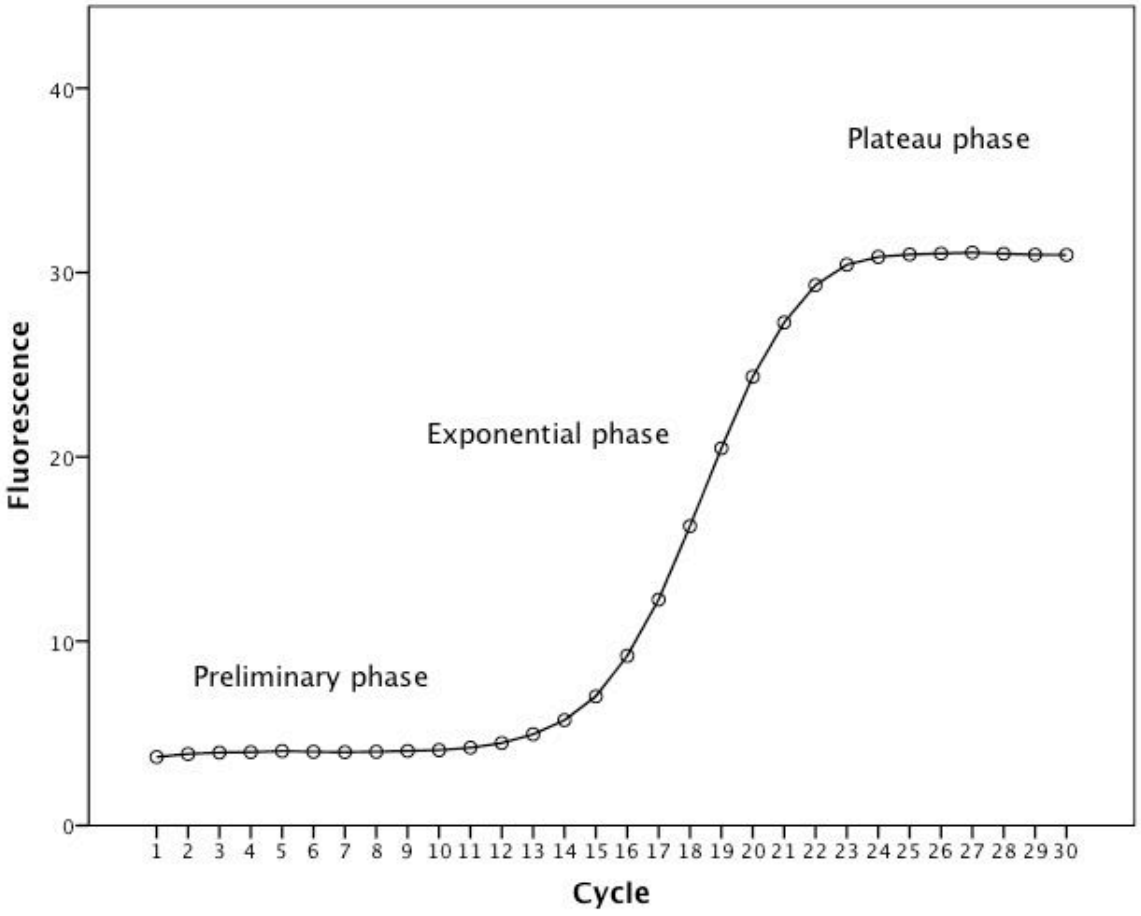

Figure 2.1 Sample amplification curve

In the preliminary phase, fluorescence from the products of the PCR reaction is insufficient to register above the baseline level (determined by the fluorescence of the 'no template' control). In the terminal plateau phase, replication rate is limited by lack of substrate. In the exponential phase, the reaction conditions

are optimal; during this phase the replication rate is fastest. The straight portion of the curve (which has the highest gradient) represents the exponential phase. Extrapolation back to the threshold fluorescence level marks the 'crossing point'. The crossing point (Cp) is the number of cycles necessary to replicate enough DNA to reach threshold fluorescence (as compared to the 'no template' control). Calculation was performed by Light Cycler LC480 software using the '2^{nd} derivative maximum' method to find the maximum rate of reaction from the fluorescence curve and extrapolate back to the Cp.

2.5.2 Analysis of PCR Plates

2.5.2.1 Assessment of the efficiency of the PCR reaction

Before proceeding to determine T/S for the experimental samples, it is necessary to verify that the telomere and 36B4 plates had similar efficiency. The Roche LightCycler 480 software constructs a standard curve using the Cp for the standard dilution samples. The slope of this curve, which would correspond to the maximum possible efficiency of reaction of 2, is -3.4. Standard curve slopes of between -2.8 and -3.8 are acceptable. Both the telomere and 36B4 plates should have standard curve slopes within this range to be comparable. The concentration of the standard calibrator according to the standard curve should be close to the expected concentration of 5ng DNA/μl.

These conditions were met for each plate analysed in order to include results from that plate. Some plates were repeated to achieve this standard.

2.5.2.2 Assessment of plate-to-plate comparability

Each plate allows the analysis of 24 samples in one experiment. Plate-to-plate comparability determines whether results obtained from individual experiments can be combined in assessing the full cohort of samples. There are two methods to assess plate-to-plate comparability. The more stringent looks at the coefficient of variation (CV) of T/S ratios for each plate under investigation. This usually produces a higher but more accurate CV.

For the data reported in this thesis:

CV = Standard deviation/Mean

%CV = Standard deviation/Mean x 100

In this case T/S ratios for 5 plates under investigation were examined.

%CV = Standard deviation/Mean x 100 = 1.016725/0.052509 x 100

%CV = 5.164527

The second method calculates the average of the standard deviations and the average of the means for the concentrations of each standard triplicate and positive control from each plate under investigation. The CV is calculated as above using the average standard deviation divided by the average mean.

For the data reported in this thesis:

“Average” standard deviation = 0.645841408

“Average” mean concentration = 20.20016116

%CV = Standard deviation/Mean x 100 = 0.645841408/20.20016116

%CV = 3.197209183

These values for plate-to-plate comparability are acceptable.

2.5.2.3 Measurement of relative telomere length (T/S)

Absolute quantification compares an experimental sample relative to standards of known concentration to provide a quantitative measure of telomere length. Relative quantification measures the Cp for one sample relative to the reference gene (in this case 36B4). Absolute quantification of experimental samples relative to the standard curve was performed for telomere and 36B4 DNA. Relative quantification of the telomere DNA relative to 36B4 DNA. These values were normalised relative to the standard calibrator to give relative telomere length (T/S) measurements which were used in further statistical evaluation.

This standard comparison means that the results could be translated into kilobasepairs (kbp) of DNA. This translation is not necessary for comparison of T/S values, but may be useful to compare these results with other studies using direct measurement of telomere length by the terminal restriction fragment length method in future analysis.

2.6 Fetuin A ELISA

Fetuin A levels in serum samples from colorectal cancer patients were analysed using the Human Fetuin-A ELISA kit (BioVendor) according to the manufacturer's instructions. Enzyme-linked immunosorbent assays (ELISA) used microplate wells coated with antibodies to fetuin A. Standard dilutions, quality controls and experimental samples were added to the wells, incubated and washed. Horseradish peroxidase (HRP)-linked antibody to fetuin A was added and bound the captured fetuin A. A further washing step ensured that only bound HRP remained to react with added substrate solution. This reaction was stopped by addition of an acidic solution after a defined time, and the amount of coloured product formed (proportional to the absorbance of the well contents) was measured by a microplate reader. The standard dilutions were used to construct a standard curve from which absorbance of experimental samples was converted into concentrations of the fetuin A.

Samples were analysed in triplicate and the coefficient of variance for each triplicate was routinely less than 6%.

2.7 Analysis of Antioxidant and Vitamin levels

After initial preparation and stabilisation as described earlier, these samples were transferred to the Department of Biochemistry at Glasgow Royal Infirmary, where further analysis was performed according to previously validated methods(Talwar, Ha et al. 1998).

2.8 Sirtuin Immunohistochemistry

2.8.1 Tissue microarray construction

Use of a tissue microarray (TMA) enabled staining of the whole patient cohort in one experiment and ensured uniformity of experimental conditions. TMAs were constructed by Mr Jonathan Platt (JP) and Ms Clare Orange, with advice from Dr Karin Oien, consultant pathologist. JP selected the tissue to be used by examination of haematoxylin and eosin (H+E) stained sections of tissue from previously diagnosed colorectal cancer patients. CO constructed the TMAs by extracting cores from tissue blocks corresponding to the areas identified on the H+E sections and mounting these in wax blocks. Four cores of 0.6mm diameter were taken from each specimen. Cores of normal tissue of various histological subtypes were included for reference and to help orient the slides.

Five TMAs were constructed to accommodate the full cohort and a small group of normal samples. Sections of thickness 2.5μm were cut and mounted on 3-aminopropylethoxysilane coated slides which were incubated at 56°C before being stored at 4°C till needed.

The layout of the TMAs is represented in Appendix 3.

2.9 Preparation of Slides for IHC

2.9.1 Dewaxing and Rehydration

Tissue was embedded in paraffin blocks to allow thin sections to be cut and mounted on slides. In order to perform IHC, the paraffin was dissolved away and the tissue rehydrated. Slides were incubated in xylene for 2x2 minutes, and then in reducing concentrations of alcohol (2x2mins in 100%, 2mins in 90%, 2mins in 70%) and finally rinsed in running tap water.

2.9.2 Antigen Retrieval

Formalin fixation of tissue involves the formation of methylene bridges within tissue proteins, which can hide antigenic epitopes. In order to retrieve (or unmask) these epitopes, i.e. break the methylene bonds, the slides were heated under pressure in a buffer solution appropriate to the antibody in use. Other methods were used in trying to optimise antibodies to SIRT1 (trypsin digestion and heating in a waterbath) but without success.

2.9.3 Blocking of Endogenous Peroxidase

As peroxidase is used to catalyse the colour change in the final step of IHC, endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 10 minutes on a stirrer. Slides were then washed in running water.

2.9.4 Blocking of Non-Specific Protein Binding

High-volume, low-affinity binding between other proteins in the tissue under investigation and the primary antibody may interfere with the staining pattern of the specific protein under investigation. To mitigate this effect, the tissue was incubated with a solution of optimum concentration of horse serum in TBS for 60 min at 25°C. The proteins in the horse serum blocked these low-affinity binding sites but did not compete with the specific binding of the primary antibody.

2.9.5 Antibody Staining

The slides were incubated with the primary antibody under optimum conditions, deduced from the preliminary optimisation experiments. These were specific to the antibody in question, and are detailed later.

2.9.6 Secondary Antibody Staining

The slides were stained with the appropriate secondary antibody system (Envision™ or LSAB) and with chromogen (diaminobenzidine, DAB), according to the manufacturer's instructions.

2.9.7 Counterstain

In order to visualise the cell components in the background, a haematoxylin counterstain was applied by incubation for 3 minutes in Harris haematoxylin, followed after rinsing by incubation in Scott's Tap Water Substitute for 2 minutes.

2.9.8 Dehydration and mounting

The slides were dehydrated by incubation in the series of increasing concentrations of alcohol (1 min in 70%, 1 min in 90%, 2x1 min in 100%) xylene (2x1min). They were mounted in di-n-butyl-phthalate in xylene (DPX) and a coverslip applied.

2.10 Sirtuin Antibodies

The antibodies used are detailed in the table below.

Sirtuin	Antibody
SIRT1	Abcam ab32441 and ab32424. Unsuccessful.
SIRT2	Lifespan Biosciences B-1565
SIRT3	Cell Signal C73E3
SIRT4	Abcam ab49173
SIRT5	Abcam ab10140
SIRT6	Lifespan Biosciences B-900
SIRT7	Abcam ab78977

Table 2.2 Antibodies used for sirtuin immunohistochemistry.

No reliable staining could be achieved for SIRT1 due to manufacturing problems, despite repeated attempts to optimize staining 'in house'. The antibodies used were validated by Western blot in-house.

2.10.1.1 Antibody Optimisation

Optimal specific conditions for IHC for each antibody were determined by experimental modification of pre-existing protocols used in this laboratory to stain other tissues with some of these antibodies. For new antibodies, experimental assessment and modification of the manufacturer's advised conditions for IHC was performed. In all cases a practice TMA using colorectal cancer tissue cores was used initially.

The specific conditions used for each antibody are summarized in the following table.

Sirtuin	Antibody reference and manufacturer	Buffer	Antibody type	Block	Blocking conditions	Optimum titre	Antibody incubation conditions	Secondary system	Secondary system conditions
SIRT2	LS-B1565, Lifespan Biosciences	TE	Polyclonal Rabbit	1.5% Normal horse serum	60 mins 25deg	1:1500	37deg, 1hr	Envision	37deg, 30mins
SIRT3	C73E3, Cell Signal	Citrate	Monoclonal Rabbit	1.5% Normal horse serum	60mins 25deg	1:50	4deg, overnight	Envision	25deg 30mins
SIRT4	ab10140, AbCam	Citrate	Polyclonal Goat	10% Normal horse serum	60mins 25deg	1:200	4deg, overnight	LSAB+	Biotin secondary antibody 30mins 25deg, Streptavidin 60 mins 25 deg
SIRT5	ab49173, AbCam	TE	Polyclonal Rabbit	1.5% Normal horse serum	20 mins 37deg	1:700	4deg, overnight	Envision	37deg, 30mins
SIRT6	LS-B900, Lifespan Biosciences	TE	Polyclonal Rabbit	1.5% Normal horse serum	20 mins 37deg	1:400	37deg, 2hrs	Envision	37deg, 30mins
SIRT7	ab78977, AbCam	TE	Polyclonal Rabbit	1.5% Normal horse serum	60mins 25deg	1:300	4deg, overnight	Envision	25deg 30mins

Table 2.3 Optimised staining conditions for each sirtuin antibody used.

2.11 Weighted Histoscore Analysis of Sirtuin-stained TMAs

Each TMA was scored by EM, according to the weighted Histoscore method described, validated and extensively used in this laboratory (Edwards, Mukherjee et al. 2004). Scoring was carried out separately for nuclei and cytoplasm. Staining was categorized as none (0), weak (1), moderate (2) or strong (3). The percentage of total tumour nuclear or cytoplasmic component falling within each category was multiplied by the weighting factor (in brackets), to give a maximum possible score of 300.

0 x percentage tumour tissue with no staining
\+ 1 x percentage tumour tissue with weak staining
\+ 2 x percentage tumour tissue with moderate staining
\+ 3 x percentage tumour tissue with strong staining
Total Histoscore (max possible score 300)

All of the cores stained for SIRT6 were double scored by Jacqueline Martin (medical student) and 10% of the cores stained for SIRT2 were rescored by EM. Inter-rater and intra-rater correlation coefficients were calculated according to standard methods in use in this laboratory (Kirkegaard, Edwards et al. 2006). Satisfactory inter-rater and intra-rater correlations were confirmed by independent assessors.

Scatterplots and Bland-Altman charts for inter-rater and intra-rater comparisons are included in Appendix 6. Inter-rater intra-class correlation coefficient (ICCC) for nuclear Histoscores was 0.851 and for cytoplasmic Histoscores was 0.913. Intra-rater intra-class correlation coefficient (ICCC) for nuclear Histoscores was 0.789 and for cytoplasmic Histoscores was 0.716.

Mean Histoscores for nuclear and cytoplasmic staining were calculated for each sample using EM's scores.

2.12 Statistical Analysis

All statistical analyses were carried out using a statistical software package (IBM SPSS Statistics 21.0).

2.12.1 Telomere Length Cohort

2.12.2 Availability of Matched Samples

Of the 97 patients who gave informed consent for the use of blood samples, one was found to have a well-differentiated neuroendocrine carcinoma involving the terminal ileum rather than the colon and was excluded from further analysis. 29 of the remaining patients had given consent for tissue deposition in the Glasgow Biobank and had sufficient surplus tissue (as judged by a consultant pathologist) to provide samples for research. In one of these cases the tumour tissue telomere length was 7.5 standard deviations greater than the mean of the rest of the samples (shown in the scatterplot below); DNA extraction from the corresponding normal colorectal tissue for this case was unsuccessful. This case was excluded from further analysis.

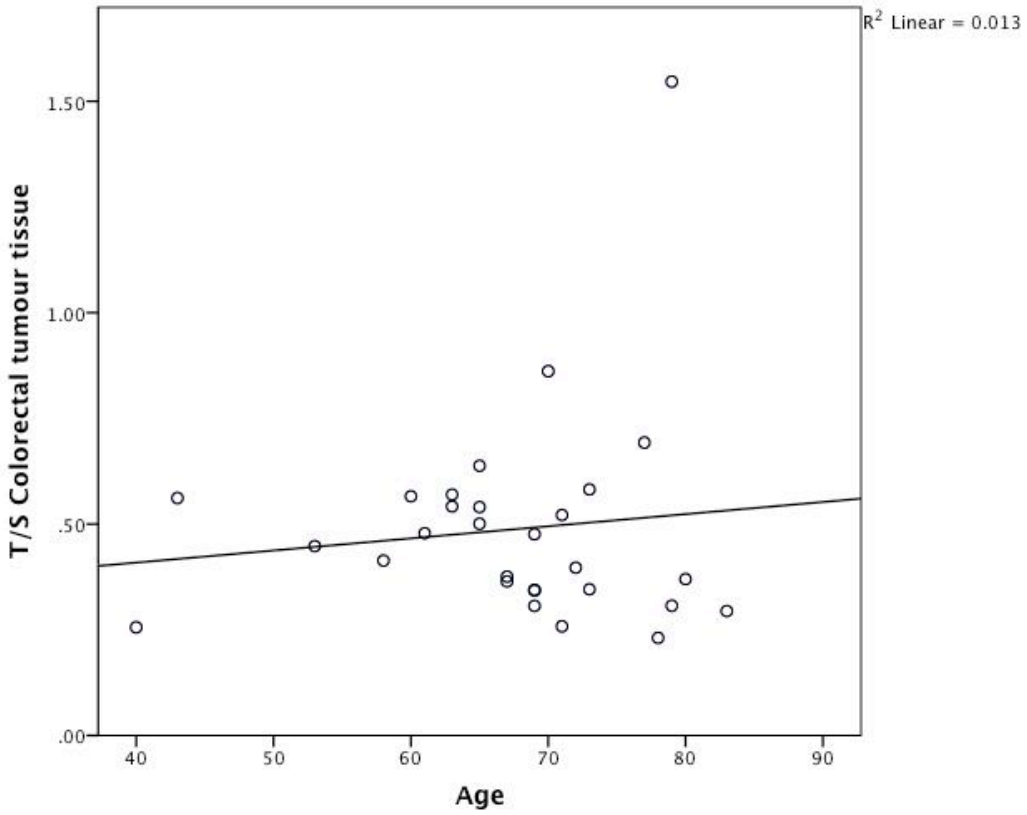

Figure 2.2 Scatterplot showing distribution of age and tumour tissue telomere length, with outlier. There is no significant correlation.

In 1 further case, DNA extraction from the normal colorectal tissue was unsuccessful and 1 other case had insufficient tumour tissue but normal colorectal tissue was provided for telomere length analysis. Thus there were 95 patients with blood available for telomere length analysis and 28 patients in the matched tissue cohort; 26 of these had tissue for both normal and colorectal tumour tissue. 27 patients had samples of normal colorectal tissue and PBLs. 27 patients had samples of colorectal tumour tissue and PBLs.

Of the 95 patients with PBLs and serum available for analysis, 4 cases did not have samples for serum fetuin A measurement. 1 of these 4 cases had both normal and colorectal tumour tissue available for telomere length analysis.

2.12.3 Statistical analysis of data from telomere length cohort

Statistical analysis for comparison of telomere lengths in matched tissue samples is detailed in the chapter discussing these results.

2.12.4 Associations with clinicopathological variables

Associations between experimental variables and clinicopathological variables were assessed. Non-parametric tests were used throughout. Correlation of continuous variables was assessed using Spearman's rho, Mann-Whitney U test was used for binary variables and Kruskal-Wallis for those with more than 2 categories. As there were multiple correlations (30) a higher p-value ($p<0.01$) was determined as the level of statistically important significance.

2.13 Statistical Analysis of Immunohistochemistry Studies

2.13.1 Availability of matched samples from normal and colorectal tumour tissue

Normal tissue was included on the TMA for 22 of the total 272 cases, although processing of the TMA resulted in included tissue for 20 -21 of these cases on some slides.

2.13.1.1 Comparison of staining in normal and tumour tissue, and survival analysis

Basic descriptive statistics were used to investigate the distribution of staining for each protein and subcellular localisation in normal and tumour tissue and to compare normal and tumour tissue. Assuming equal distribution of deaths in each group as the null hypothesis, the cohort was divided into quartiles by protein Histoscore for each combination of protein (SIRT2-7) and subcellular location (nuclear or cytoplasmic) Kaplan-Meier survival curves were produced for each combination. Equality of survival distribution was compared using the log rank (Mantel-Cox) test. Univariate and multivariate analysis using Cox (proportional hazard) regression was performed to investigate significant influences on survival.

2.13.2 Associations with clinicopathological variables

Associations between protein expression and clinicopathological variables were assessed. Correlation of Histoscores with clinicopathological factors known to be of relevance in colorectal cancer was assessed using nonparametric tests throughout (Mann-Whitney U test for binary variables and Kruskal-Wallis for those with more than 2 categories). Correlation of continuous variables was assessed using Spearman's rho. As there were multiple correlations (22) a higher p-value ($p<0.01$) was determined as the level of statistically important significance.

2.14 Sirtuin Interrelationships: Statistical Considerations

Previous data on intercorrelation of normal sirtuin RNA levels and intercorrelation of tumour sirtuin RNA levels comes from a different population of colorectal cancer patients published in the MD thesis 'The influence of biological ageing in the pathogenesis of colorectal cancer' by F Maxwell (Maxwell 2013). The Pearson correlation coefficient was quoted, implying normal distribution of the data.

2.14.1 Appropriate level of significance for correlations

The correlations between levels of expression of sirtuin RNAs were compared with the correlations between levels of expression of sirtuin proteins. This was complicated by the measurement of Histoscores for two subcellular locations (nuclear and cytoplasmic) for the proteins. In keeping with the statistical

approach in the rest of this thesis, (using nonparametric tests throughout) only Spearman correlations were used. For correlations between sirtuin Histoscores in the two subcellular locations, the total number of correlation tests performed is 66 for tumour tissue and 55 for normal tissue. (There was no positive staining for SIRT5 in the nucleus in normal tissue, so there are no correlation tests with SIRT5N for normal tissue). A p-value less than 0.01 was accepted as the level of statistically important significance for correlations. This is a higher level than was accepted in the previous data on correlations between levels of expression of sirtuin RNAs, although the correlation used was the Pearson correlation. The comparisons examine the networks of correlations at RNA and protein level rather than the individual correlations, for which the different analyses are used. In order to compare like with like, RNA correlations with p-value <0.01 were included in this comparative analysis.

2.14.2 Correlation coefficient and p-value

In assessing correlations, the correlation coefficient must also be taken into account, as correlation coefficients <0.35 in magnitude are generally deemed weak, and correlation coefficients >0.65 in magnitude, strong. It is possible to have a strong correlation that is not statistically significant. Likewise, statistically significant correlations may not be very strong. The interpretation of these two parameters must be made in context of information on interactions between the correlated variables. In the case of sirtuin interactions, such information is sparse at present. It is described further in the relevant results chapter.

2.14.3 Correlations between RNA expression of different sirtuins in normal colorectal tissue

Correlations in RNA expression in normal colorectal tissue with p<0.01 are indicated in the table below with green squares. This table is adapted with permission from ‘The influence of biological ageing in the pathogenesis of colorectal cancer’, MD Thesis, University of Glasgow (Maxwell 2013).

	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1						
SIRT2						
SIRT3						
SIRT4						
SIRT5						
SIRT6						
SIRT7						

Table 2.4 Correlations in RNA expression in normal colorectal tissue with p<0.01 are indicated with green squares. This table is adapted with permission from 'The influence of biological ageing in the pathogenesis of colorectal cancer', MD Thesis, University of Glasgow (Maxwell 2013).

2.14.4 Correlations between RNA expression of different sirtuins in colorectal tumour tissue

Correlations in RNA expression in colorectal tumour tissue with p<0.01 are indicated in the table below with red squares. This table is adapted with permission from 'The influence of biological ageing in the pathogenesis of colorectal cancer', MD Thesis, University of Glasgow (Maxwell 2013).

	SIRT2	SIRT3	SIRT4	SIRT5	SIRT6	SIRT7
SIRT1						
SIRT2						
SIRT3						
SIRT4						
SIRT5						
SIRT6						
SIRT7						

Table 2.5 Correlations in RNA expression in colorectal tumour tissue with p<0.01 are indicated with red squares. This table is adapted with permission from 'The influence of biological ageing in the pathogenesis of colorectal cancer', MD Thesis, University of Glasgow (Maxwell 2013).

2.14.5 Correlations between protein expression of different sirtuins in normal colorectal tissue

Correlations between sirtuin/localisation combinations in normal colorectal tissue samples were examined. A p-value of 0.01 was accepted as statistically

significant. These correlations are indicated in the table below with red background.

		Normal SIRT 2 Mean Histoscore (Nuclei)	Normal SIRT 2 Mean Histoscore (Cytoplasm)	Normal SIRT 3 Mean Histoscore (Nuclei)	Normal SIRT 3 Mean Histoscore (Cytoplasm)	Normal SIRT 4 Mean Histoscore (Nuclei)	Normal SIRT 4 Mean Histoscore (Cytoplasm)	Normal SIRT 5 Mean Histoscore (Nuclei)	Normal SIRT 5 Mean Histoscore (Cytoplasm)	Normal SIRT 6 Mean Histoscore (Nuclei)	Normal SIRT 6 Mean Histoscore (Cytoplasm)	Normal SIRT 7 Mean Histoscore (Nuclei)	Normal SIRT 7 Mean Histoscore (Cytoplasm)
Normal SIRT 2 Mean Histoscore (Nuclei)	Correlation Coefficient	1.000	.258	-.515*	.229	.473*	.204		-.344	-.160	-.319	.494*	.683**
	Sig. (2-tailed)		0.25905171	0.01684037	0.31719032	0.03018218	0.37438564		0.13734272	0.49951340	0.17072109	0.02284917	0.00065208
	N	21	21	21	21	21	21	20	20	20	20	21	21
Normal SIRT 2 Mean Histoscore (Cytoplasm)	Correlation Coefficient		1.000	-.315	-.210	.079	.026		.168	-.326	.003	.271	-.161
	Sig. (2-tailed)			0.16478125	0.36002887	0.73240624	0.91114317		0.47861634	0.16068484	0.99114458	0.23475101	0.48556225
	N		21	21	21	21	21	20	20	20	20	21	21
Normal SIRT 3 Mean Histoscore (Nuclei)	Correlation Coefficient			1.000	.130	-.186	.218		.563**	.436	.050	-.138	-.058
	Sig. (2-tailed)				0.56425762	0.40618851	0.32896873		0.00974608	0.05487084	0.83531378	0.53932690	0.79895652
	N			22	22	22	22	20	20	20	20	22	22
Normal SIRT 3 Mean Histoscore (Cytoplasm)	Correlation Coefficient				1.000	.245	.442*		.313	.246	-.150	.124	.581**
	Sig. (2-tailed)					0.27181124	0.03952189		0.17855114	0.29524638	0.52846796	0.58114663	0.00461773
	N				22	22	22	20	20	20	20	22	22
Normal SIRT 4 Mean Histoscore (Nuclei)	Correlation Coefficient					1.000	.576**		.207	.086	-.500*	.256	.157
	Sig. (2-tailed)						0.00501604		0.38219090	0.71733897	0.02470505	0.24966014	0.48577449
	N					22	22	20	20	20	20	22	22
Normal SIRT 4 Mean Histoscore (Cytoplasm)	Correlation Coefficient						1.000		.371	.279	-.297	.236	.195
	Sig. (2-tailed)								0.10750844	0.23350537	0.20322531	0.29134365	0.38354247
	N						22	20	20	20	20	22	22
Normal SIRT 5 Mean Histoscore (Nuclei)	Correlation Coefficient												
	Sig. (2-tailed)												
	N							20	20	20	20	20	20
Normal SIRT 5 Mean Histoscore (Cytoplasm)	Correlation Coefficient								1.000	.195	-.024	-.156	-.291
	Sig. (2-tailed)									0.40913607	0.91961890	0.51252191	0.21256507
	N								20	20	20	20	20
Normal SIRT 6 Mean Histoscore (Nuclei)	Correlation Coefficient									1.000	.367	.058	.038
	Sig. (2-tailed)										0.11096973	0.80771043	0.87318218
	N									20	20	20	20
Normal SIRT 6 Mean Histoscore (Cytoplasm)	Correlation Coefficient										1.000	-.176	-.233
	Sig. (2-tailed)											0.45665962	0.32270038
	N										20	20	20
Normal SIRT 7 Mean Histoscore (Nuclei)	Correlation Coefficient											1.000	.450*
	Sig. (2-tailed)												0.03576067
	N											22	22
Normal SIRT 7 Mean Histoscore (Cytoplasm)	Correlation Coefficient												1.000
	Sig. (2-tailed)												
	N												22

Table 2.6 Correlations between Histoscores for each combination of sirtuin and subcellular localisation in normal colorectal tissue. Red cells indicate combinations with p<0.01.

2.14.6 Correlations level of significance for correlations between protein expression of different sirtuins in colorectal tumour tissue

Correlations between sirtuin/localization combinations in colorectal tumour tissue were examined. A p-value of 0.01 was accepted as statistically significant. Correlations with p<0.01 and correlation co-efficient >0.35 are indicated in the table below with red background. There were several correlations with p<0.01 and weak correlation coefficients. Correlations with p<0.01 and correlation co-efficient <0.35 are indicated in the table below with orange background.

		Tumour SIRT2 Mean Histoscore (Nuclei)	Tumour SIRT2 Mean Histoscore (Cytoplasm)	Tumour SIRT3 Mean Histoscore (Nuclei)	Tumour SIRT3 Mean Histoscore (Cytoplasm)	Tumour SIRT4 Mean Histoscore (Nuclei)	Tumour SIRT4 Mean Histoscore (Cytoplasm)	Tumour SIRT5 Mean Histoscore (Nuclei)	Tumour SIRT5 Mean Histoscore (Cytoplasm)	Tumour SIRT6 Mean Histoscore (Nuclei)	Tumour SIRT6 Mean Histoscore (Cytoplasm)	Tumour SIRT7 Mean Histoscore (Nuclei)	Tumour SIRT7 Mean Histoscore (Cytoplasm)
Tumour SIRT2 Mean Histoscore (Nuclei)	Correlation Coefficient	1.000	.253**	.066	-.003	.241**	-.020	.000	-.141*	.128*	-.125*	.234**	-.058
	Sig. (2-tailed)		0.00002813	0.28675450	0.96744814	0.00007120	0.74673546	0.99466348	0.02207710	0.03916414	0.04240744	0.00012232	0.34337191
	N	267	267	266	266	266	266	265	265	262	262	265	265
Tumour SIRT2 Mean Histoscore (Cytoplasm)	Correlation Coefficient		1.000	-.206**	.339**	.085	.470**	-.038	.181**	-.157*	.351**	-.077	.205**
	Sig. (2-tailed)			0.00072281	0.00000001	0.16865287	0.00000000	0.54036548	0.00317293	0.01090360	0.00000001	0.21404104	0.00079613
	N		267	266	266	266	266	265	265	262	262	265	265
Tumour SIRT3 Mean Histoscore (Nuclei)	Correlation Coefficient			1.000	-.235**	.256**	-.248**	.210**	.011	.466**	-.301**	.265**	.011
	Sig. (2-tailed)				0.00010933	0.00002332	0.00004163	0.00058052	0.86061763	0.00000000	0.00000069	0.00001231	0.85919518
	N			267	267	267	267	265	265	262	262	265	265
Tumour SIRT3 Mean Histoscore (Cytoplasm)	Correlation Coefficient				1.000	-.016	.563**	.004	.156*	-.148*	.460**	-.040	.321**
	Sig. (2-tailed)					0.79888735	0.00000000	0.94790442	0.01091280	0.01638800	0.00000000	0.51560479	0.00000009
	N				267	267	267	265	265	262	262	265	265
Tumour SIRT4 Mean Histoscore (Nuclei)	Correlation Coefficient					1.000	.110	.152*	.087	.279**	-.025	.121*	-.043
	Sig. (2-tailed)						0.07114308	0.01275253	0.15567146	0.00000439	0.68796835	0.04883032	0.48778645
	N					270	270	267	267	262	262	265	265
Tumour SIRT4 Mean Histoscore (Cytoplasm)	Correlation Coefficient						1.000	-.009	.274**	-.123*	.438**	-.160**	.284**
	Sig. (2-tailed)							0.88315279	0.00000535	0.04686922	0.00000000	0.00886534	0.00000258
	N						270	267	267	262	262	265	265
Tumour SIRT5 Mean Histoscore (Nuclei)	Correlation Coefficient							1.000	-.140*	.231**	-.014	.061	.071
	Sig. (2-tailed)								0.02224058	0.00015750	0.81559412	0.32504268	0.25249582
	N							268	268	263	263	263	263
Tumour SIRT5 Mean Histoscore (Cytoplasm)	Correlation Coefficient								1.000	-.024	.189**	-.063	.218**
	Sig. (2-tailed)									0.70106867	0.00204316	0.30741700	0.00036046
	N								268	263	263	263	263
Tumour SIRT6 Mean Histoscore (Nuclei)	Correlation Coefficient									1.000	-.274**	.305**	-.094
	Sig. (2-tailed)										0.00000657	0.00000054	0.13017869
	N									263	263	260	260
Tumour SIRT6 Mean Histoscore (Cytoplasm)	Correlation Coefficient										1.000	-.220**	.335**
	Sig. (2-tailed)											0.00034230	0.00000003
	N										263	260	260
Tumour SIRT7 Mean Histoscore (Nuclei)	Correlation Coefficient											1.000	.197**
	Sig. (2-tailed)												0.00125036
	N											266	266
Tumour SIRT7 Mean Histoscore (Cytoplasm)	Correlation Coefficient												1.000
	Sig. (2-tailed)												
	N												266

Table 2.7 Correlations between sirtuin/localization combinations in colorectal tumour tissue were examined. A p-value of 0.01 was accepted as statistically significant. Correlations with p<0.01 and correlation co-efficient >0.35 are indicated in the table below with red background. Correlations with p<0.01 and correlation co-efficient <0.35 are indicated in the table below with orange background.

Only 6 of the 36 correlations between sirtuin protein levels identified as statistically significant in tumour tissue have correlation coefficients greater than 0.35.

Only correlations which were both strong and statistically significant were used to build networks representing associations between sirtuins at mRNA and protein levels. These correlations could be a proxy measure of sirtuin protein interaction, although the interaction may be indirect.

3 Fetuin A, Inflammation and Prognostic Markers in Colorectal Cancer

3.1 Introduction

Fetuin A is a negative acute phase serum protein produced in the liver. It is pertinent to telomere biology in chronic kidney disease as mortality associated with short telomeres in PBLs is dependent on fetuin A levels (Carrero, Stenvinkel et al. 2008). This relationship is independent of inflammatory status. More recent work has highlighted that in colorectal cancer, a very different age-associated disease, short telomeres were associated with lower fetuin A levels (Monaghan, Maxwell et al. 2010; Maxwell, McGlynn et al. 2011). This may reflect loss of systemic capacity for redox homeostasis.

Associations between serum fetuin A and accepted markers of prognostic significance in colorectal cancer were evaluated in a cohort of colorectal cancer patients.

Clinical and Pathological Characteristics of the Patient Cohort

Clinical and pathological characteristics of this patient cohort are summarised in the table below. The total cohort numbers 95, with fetuin A measurements for 91.

Sex	M	42	**Dukes' code**	NTF	5
	F	53		A	18
Age	<40	2		B	36
	40-49	4		C	25
	50-59	16		D	7
	60-69	33		Not recorded	4
	70-79	25		**Total**	**95**
	>80	15	**T stage**	NTF	5
	Total	**95**		1	11
BMI	<20	3		2	11
	20-25	30		3	48
	25.1-30	29		4	14
	30.1-35	11		Not recorded	6
	>35	12		**Total**	**95**
	Not recorded	10	**N stage**	0	60
	Total	**95**		1	19
Albumin	<35	26		2	10
	>35	68		Not recorded	6
	Not recorded	1		**Total**	**95**
	Total	**95**	**AJCC stage**	NTF	5
CRP	>10	74		1	18
	<10	20		2	35
	Not recorded	1		3	26
	Total	**95**		4	6
mGPS	0	74		Not recorded	5
	1	8		**Total**	**95**
	2	12	**Peritoneum involved**	Y	9
	Not recorded	1		N	78
	Total	**95**		**Total**	**87**
Smoking history	N	49	**Margin involved**	Y	6
	Y	14		N	81
	Ex	32		**Total**	**87**
	Total	**95**	**Venous invasion**	Y	46
Tumour location	Colon	59		N	40
	Rectum	36		**Total**	**86**
	Total	**95**	**Tumour perforation**	Y	3
Differentiation	Poor	4		N	84
	Well/Mod	79		**Total**	**87**
	NTF	4	**High risk Petersen Index**	Y	52
	Not recorded	8		N	48
	Total	**95**		**Total**	**90**

Table 3.1 Clinicopathological characteristics of the patient cohort for measurement of telomere lengths and serum fetuin A.

3.2 Overview of serum fetuin A and clinicopathological factors

Associations between fetuin A and 22 clinicopathological factors are summarised in the following tables. p<0.01 was accepted as the level of statistical significance; associations with p-value >0.01 are lowlighted in grey text. Significant associations are subsequently discussed in more detail.

		Serum fetuin A
Demographic variables	**Age**	Weak inverse relationship, Spearman rank correlation coefficient -0.211, p=0.045. Pearson Chi square, p=0.197
	Sex	Pearson Chi square, p=0.333
	Weight	Fetuin A below the median is associated with lower weight, Pearson Chi square, p=0.024
	BMI	Fetuin A below the median is associated with lower BMI. Pearson Chi square, p=0.044, Linear by-Linear Association, p=0.370
Inflammatory markers	**WCC**	Inverse relationship, Spearman rank correlation coefficient -0.348, p=0.00084.
	CRP	Inverse relationship, Spearman rank correlation coefficient -0.368, p=0.00043.
	Albumin	Direct relationship, Spearman rank correlation coefficient 0.369, p=0.00035
	mGPS	High fetuin A is associated with lower mGPS, Kruskal-Wallis Test, p=0.001
Atypical markers of inflammation	**Hb**	Direct relationship, Spearman rank correlation coefficient 0.385, p=0.00019.
	Plt	Weak inverse relationship, Spearman rank correlation coefficient -0.282, p=0.007. Lower fetuin A is associated with platelet count above the median. Mann-Whitney U test, p=0.016
	Smoking status	Fetuin A levels are higher in never smokers, Mann-Whitney U test, p=0.034
Serum antioxidant levels	**Lutein**	Pearson Chi square, p=0.697
	Lycopene	Pearson Chi square, p=0.897
	alpha-carotene	Pearson Chi square, p=0.129
	beta-carotene	Pearson Chi square, p=0.591
	VitA	Pearson Chi square, p=0.897
	VitE	Pearson Chi square, p=0.172
Total comorbidities		Fetuin A below the median is associated with more comorbidities. Pearson Chi square, p=0.195, Linear-by-Linear Association, p=0.036
Tumour location		Pearson Chi square, p=0.573
Telomere length	**Leukocytes**	Pearson Chi square, p=0.173
	Normal colorectal tissue	Pearson Chi square, p=1.000
	Colorectal tumour tissue	Highest quartile telomere length is associated with highest quartile fetuin A, Pearson Chi square, p=0.029

Table 3.2 Associations between serum fetuin A and demographic variables and markers of inflammatory status. p<0.01 was accepted as the level of statistical significance. Associations with higher p-value are lowlighted.

		Serum fetuin A
Measures of cancer staging	**Dukes' stage**	Fetuin A above median is asssociated with lower Dukes'stage. Pearson Chi square, p=0.195, Linear-by-Linear Association, p=0.036
	AJCC overall stage	Fetuin A above median is asssociated with lower AJCC stage. Pearson Chi square, p=0.034, Linear-by-Linear Association, p=0.030
	T stage	Fetuin A above median is asssociated with higher T stage. Pearson Chi square, p=0.028, Linear-by-Linear Association, p=0.008
	N stage	Pearson Chi square, p=0.634
	M stage	Pearson Chi square, p=0.457
Pathological prognostic markers	**Differentiation**	Fetuin A above the median is associated with poor differentiation, Pearson Chi square, p=0.033
	Margin involvement	Pearson Chi square, p=0.747
	Peritoneal involvement	Pearson Chi square, p=0.871
	Tumour perforation	Pearson Chi square, p=0.061
	Venous invasion	Pearson Chi square, p=0.261
	High risk Petersen index	Pearson Chi square, p=0.244

Table 3.3 Associations between serum fetuin A and markers of histopathological prognostic significance. p<0.01 was accepted as the level of statistical significance. Associations with higher p-value are lowlighted.

3.2.1 Inflammatory status

3.2.1.1 Markers of classical systemic inflammatory response

Serum fetuin A levels correlated with white cell count, serum CRP and albumin, as expected since it is an acute phase protein. Correlation coefficients were of moderate strength, although statistically significant to a high degree.

	Spearman rank correlation coefficient	p value
WCC	-0.348	0.00084
CRP	-0.368	0.00043
Albumin	0.369	0.00035

Table 3.4 Spearman rank correlation coefficients and associated p-values, summarising correlations between serum fetuin A and markers of inflammation.

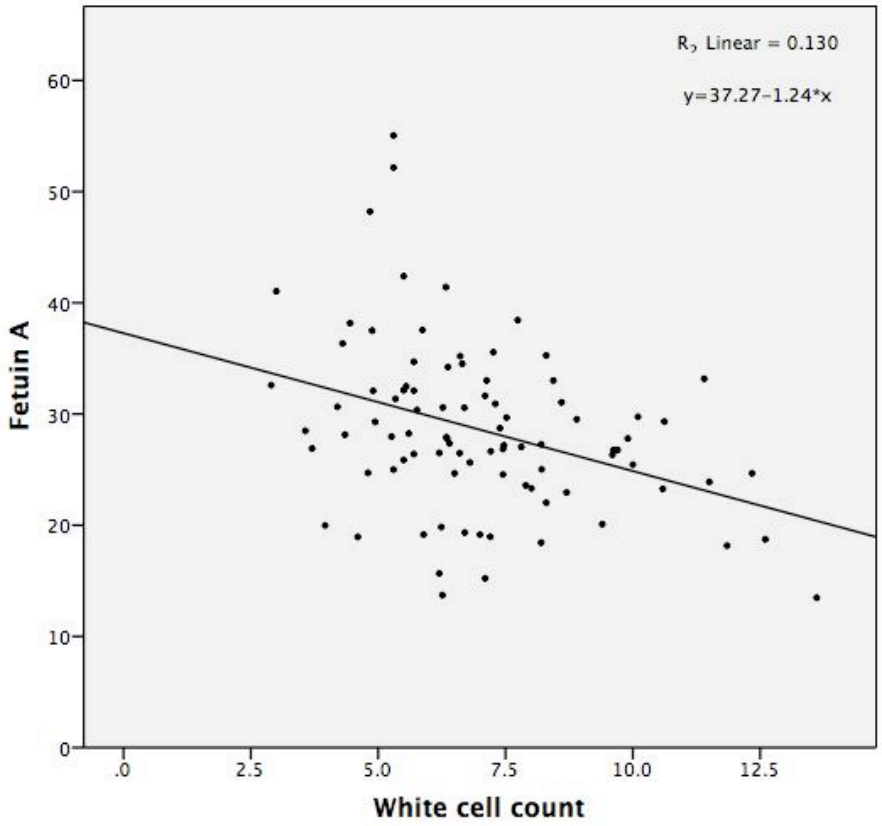

Figure 3.1 Scatterplot of serum fetuin A level with white cell count, including line of best fit, showing the moderate inverse correlation between the two (Spearman rank correlation coefficient -0.348, p=0.00084).

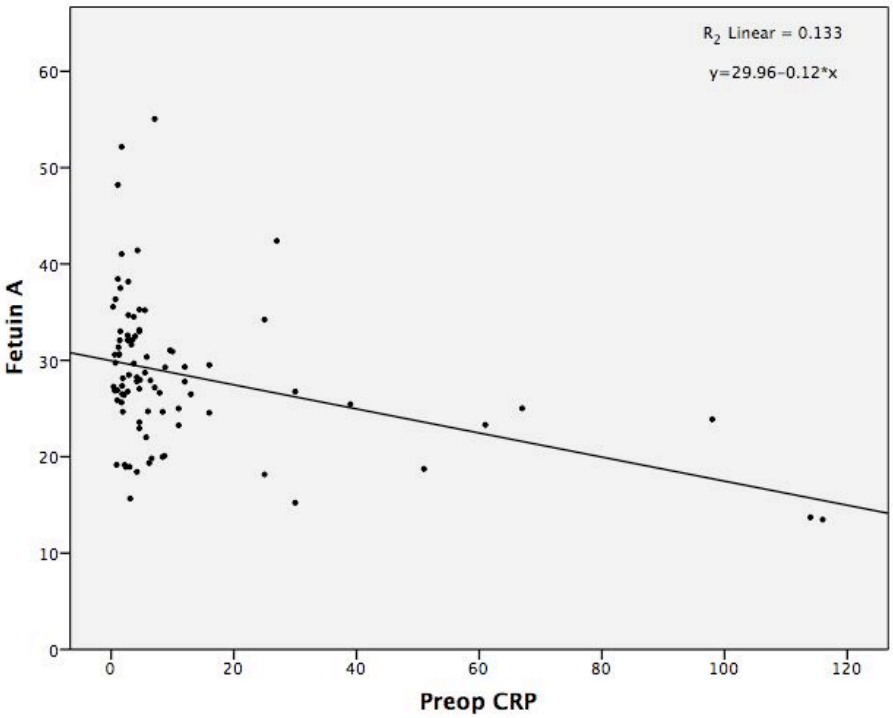

Figure 3.2 Scatterplot of serum fetuin A level with preoperative CRP, including line of best fit, showing the moderate inverse correlation between the two (Spearman rank correlation coefficient -0.368, p=0.00043).

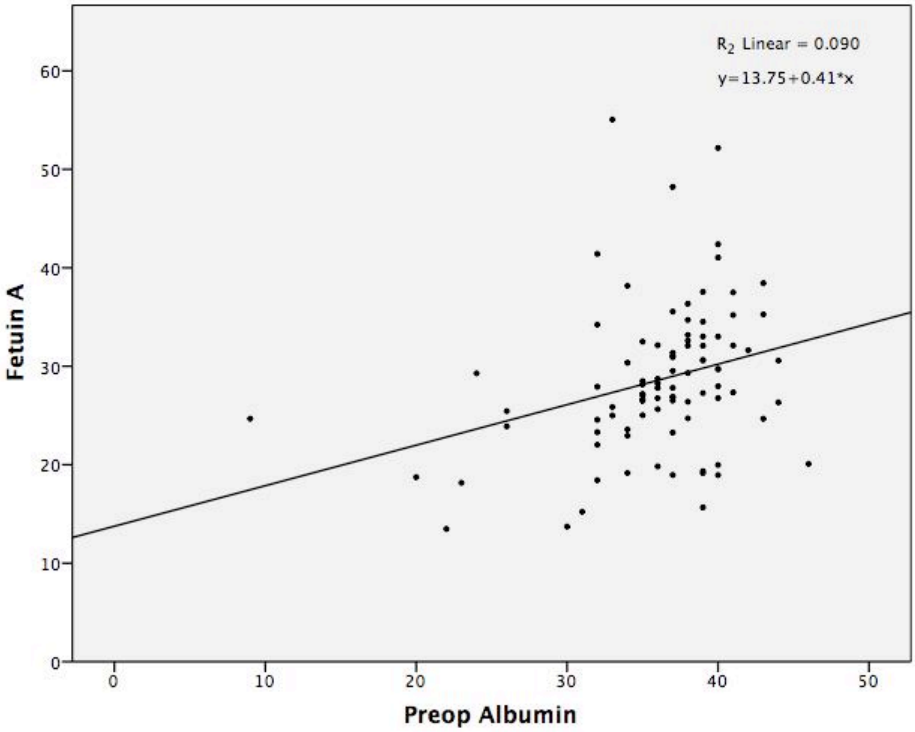

Figure 3.3 Scatterplot of serum fetuin A level with serum albumin, including line of best fit, showing the moderate positive correlation between the two (Spearman rank correlation coefficient 0.369, p=0.00035).

High serum fetuin A is associated with mGPS (Kruskal-Wallis Test, p=0.001).

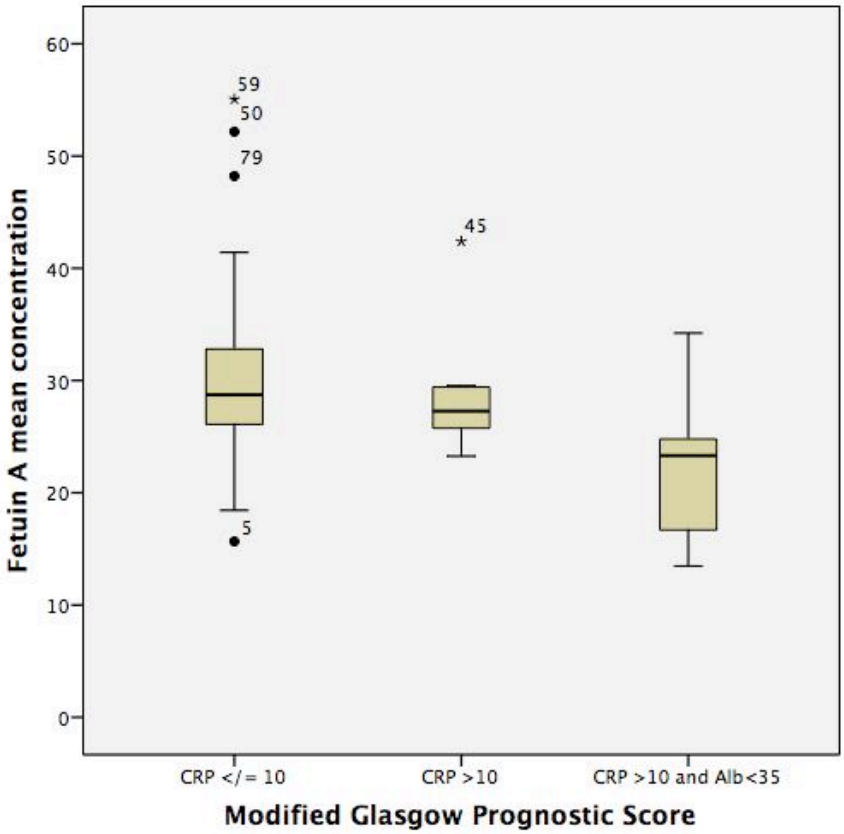

Figure 3.4 Boxplot showing serum fetuin A data grouped by mGPS score. Higher fetuin A was significantly associated with lower mGPS (Kruskal-Wallis Test, p=0.001).

3.2.1.2 Association with haemoglobin and platelet count

Serum fetuin A showed significant correlation with the atypical inflammatory markers haemoglobin and platelet count. The correlation with haemoglobin was much stronger.

	Spearman rank correlation coefficient	p value
Haemoglobin	0.385	0.00019
Platelet count	-0.282	0.0074

Table 3.5 Spearman rank correlation coefficients and associated p-values, summarising correlations between serum fetuin A and atypical inflammatory markers.

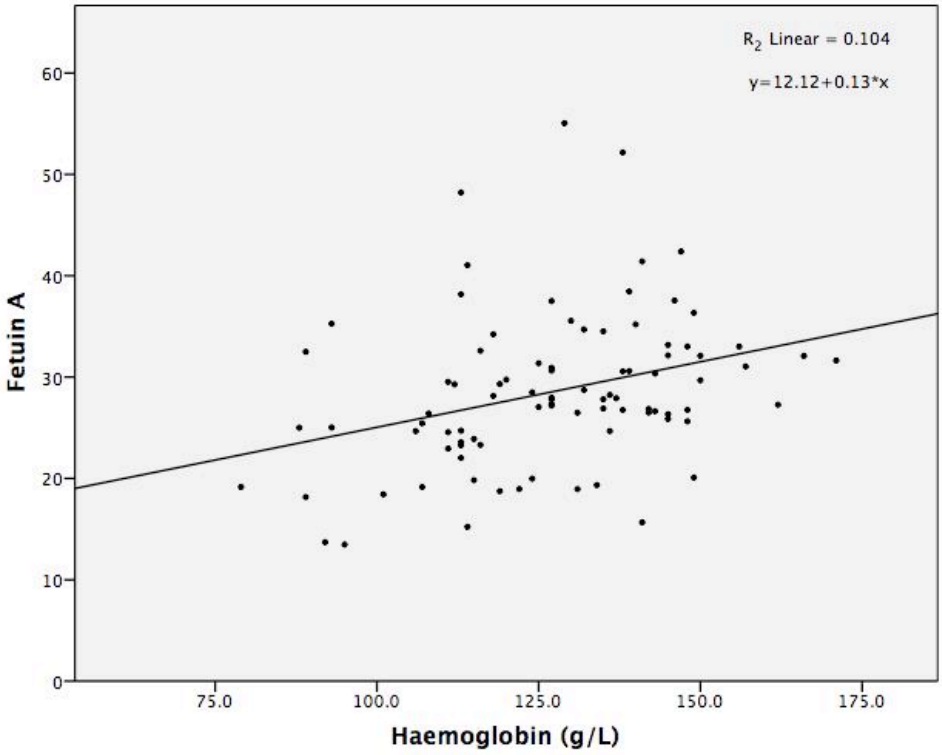

Figure 3.5 Scatterplot of serum fetuin A level with haemoglobin, including line of best fit, showing the moderate correlation between the two (Pearson correlation coefficient= 0.322, p=0.0021).

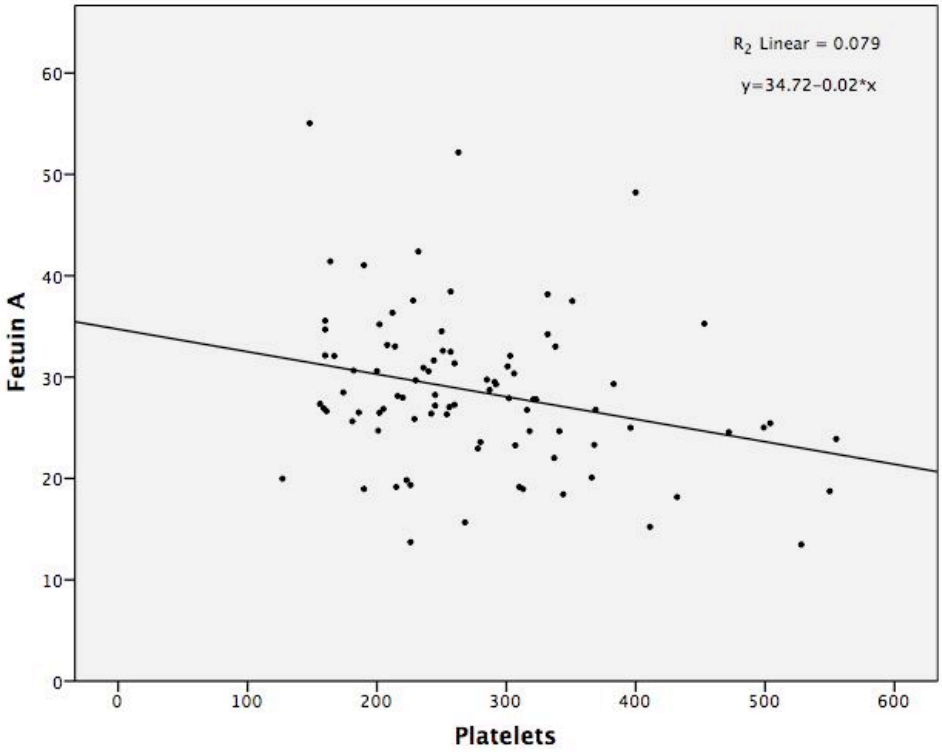

Figure 3.6 Scatterplot of serum fetuin A level with haemoglobin, including line of best fit, showing the relatively weak correlation between the two (Spearman's rho= -0.282, p=0.0074).

3.2.2 Pathological prognostic factors

3.2.2.1 Association with tumour stage

T stage measures local invasion of tumour. Lower fetuin A was associated with higher T stage (Pearson Chi square, p=0.028, linear-by-linear association, p=0.008).

		Fetuin A		
		Below median	Above median	Total
T stage	No tumour	0	5	5
	Confined to submucosa	4	7	11
	Muscularis propria	2	9	11
	Pericolorectal tissues	27	20	47
	Through serosa and/or beyond	6	5	11
Total		39	46	85

Table 3.6 Chi square analysis of fetuin A categorised about the median with respect to T stage. Lower fetuin A levels were more likely to be associated with higher T stage (Pearson Chi square, p=0.028, linear-by-linear association, p=0.008).

3.3 Discussion

3.3.1 Fetuin A and age

The inverse correlation with age shown before in humans (Maxwell 2013) is not statistically significant here although a similar trend is seen. The age range of patients in this study was 32-94, a wider range than Maxwell reported. Fetuin A levels are typically very high in neonatal life and decrease after birth. One study investigated levels in a cohort of the very old (Bellia, Tomaiuolo et al. 2012). These authors found that in 256 subjects aged 81-100 years, fetuin A levels were positively correlated with age. There are no definitive accounts of fetuin A variation throughout adult life, nor any correlation with lifespan in younger adults. Different trajectories of fetuin A variation with time may differentiate those with a longer lifespan and a larger analysis over the range of adult life would be informative.

3.3.2 Fetuin A and inflammation

Fetuin A has a central, although not straightforward, role in the promulgation of the inflammatory response and this is evident in the range of associations with specific markers of inflammation, and with smoking. The associations with other atypical markers of inflammation suggests that fetuin A might also provide a reflection of the resilience of the organism, in terms of the ability to cope with the demands of the systemic inflammatory response over the longer term and to effect significant repair.

Survival data for this cohort is not yet available, but it will be interesting to observe whether the poor prognosis associated with systemic inflammation is specifically related to fetuin A levels as well.

3.3.3 Fetuin A and telomere length

One of the aims of this project was to investigate a possible relationship between telomere length in PBLs and colorectal tissue and serum fetuin A. Unfortunately, this experiment had very low power, due to the small number of incident cases of colorectal cancer within the time period for sample collection. This meant that it was not possible to comment definitively on any relationship

between fetuin A and telomere lengths in PBLs, or gut tissue. Previous associations reported from this laboratory cannot be corroborated, nor is there strong evidence to refute them.

Telomere lengths in PBLs tend to be shorter in patients with inflammatory conditions, including renal failure. It would be expected that an appropriately powered study would demonstrate an association between low fetuin A levels and short telomeres.

Likewise, the relationship between telomere length in gut tissue and fetuin A may well shed interesting light on the relationship between these two parameters. At present, one study has shown that colorectal epithelium binds fetuin A and colorectal tumour binds less fetuin A than normal mucosa (Swallow, Partridge et al. 2004). Information on the binding substrate, and intracellular effects is limited. Telomere length is known to be vulnerable to damage by ROS and so the reduced activity of fetuin A in inflammation may exacerbate this situation, but further comment is not possible on the basis of available data.

3.3.4 Fetuin A and cancer

Decreasing fetuin A levels are associated with higher T stage in this dataset. This is the first demonstration of an association between serum fetuin A and cancer stage in colorectal cancer. No separate association with nodal status was evident.

In contrast to the situation in most other cancers studied, there is no clear evidence of reduced levels of serum fetuin A in colorectal cancer. One study shows decreased fetuin A on serum proteomic analysis, in carpeting FAP patients (n=3) compared to diffuse FAP patients (n=5) or normal cases (n=8) (Quaresima, Crugliano et al. 2008). Diffuse FAP shows less severe polyposis. Although FAP increases the risk of development of colorectal cancer (and other forms of cancer) significantly, all of these patients had already undergone prophylactic colectomy; none had a diagnosis of colorectal cancer.

This study also analysed serum from 3 sporadic cases of colorectal cancer and 2 with HNPCC, and these did not show the variations in fetuin A seen in the FAP

cases. Fetuin A inhibits TGFβ, which in turn modulates the Wnt pathway, by targeting its components including APC, which is the gene mutated in FAP. This may explain the particular interaction between fetuin A and FAP. APC mutations are common in colorectal cancers generally however, so that it should not exclude a general effect of fetuin A on sporadic colorectal cancers. Admittedly the numbers of cases in each category in this report were low.

Another paper showed no significant difference between serum fetuin A levels in 31 cases with cancer and 30 controls, (Dowling, Clarke et al. 2011). In this report, cases with cancer had tumours of AJCC stages 3b-4 so they were of higher stage generally than the cases in this cohort. The number of cases was much smaller and the variation in fetuin A levels within the cancer group was higher than either normal males or normal females. Still the mean and median serum fetuin A of the cancer groups tended to be lower than in normal samples, although this difference did not reach statistical significance.

As mentioned above, fetuin A binds to colorectal epithelium, and binding was reduced in colorectal cancer(Swallow, Partridge et al. 2004). With the data provided here, correlation of fetuin A immunohistochemical staining in colorectal tissue, serum fetuin A and colorectal cancer stage would be ideal to investigate the relationship further.

3.3.5 Fetuin A as a biomarker of ageing

Low serum fetuin A is associated with several age-associated diseases. There was no significant association with previous history of cancer or with total comorbidities in this cohort, likely because of the relatively small numbers of cases. The trend in association with numbers of comorbidities could suggest an integration of information about previous illness, and insults to the structural integrity of the organism – cumulative ageing.

An integrated summary of the effects of multiple disease processes within one organism, a valid marker of resilience, would be ideal characteristics of a possible surrogate marker for biological age. However, in order to investigate this possibility, the essential starting point would be a good understanding of the variation in fetuin A levels with chronological age.

The possibility alluded to above that fetuin A differentiates those with long lifespan would be of great interest to the study of fetuin A as a biomarker of ageing. Given the variation (both up and down) of fetuin A levels with acute influences (e.g. acute inflammation) is it possible that it may exhibit a more general drift in response to more gradual processes such as biological ageing? Does a Biomarker of ageing necessarily follow a unidirectional course? Could a Biomarker of Ageing identify a person as becoming biologically younger as well as older, or as ageing at a variable rate?

Certainly the original Baker and Sprott definition referred to 'physiological (functional) age' – and does not exclude regaining of function with the passage of time. If repair processes were sufficiently time-consuming, and complete, as to allow us to measure function during the repair process as well as after it, there is no reason why such a re-gain of function should not be observed. This may be a question of scale of measurement of biomarker as well as scale of repair, and at present large scale repair of a severely damaged organ is not within our power, or normal physiological process. Measurement of the rate of ageing or deterioration may be a more realistic aim.

McClearn acknowledges 'continuities and discontinuities in the subprocesses of ageing' which could describe the utility of fetuin A as a biomarker of ageing. Measurements taken in the context of acute self-limiting inflammatory episodes would give an inaccurate estimate but observation of longer-term trends may be useful for assessment of individuals if we have a framework of reference. McClearn also allows for the possibility that biomarkers may change through the course of an organism's life. It is possible that the relationship between fetuin A and renal function described in the study referred to above(Bellia, Tomaiuolo et al. 2012) offers such an example. Renal function declines with age and this continues in the very old. Fetuin A on the other hand, which seems to decline with age in an unselected population over most of the adult age range, increases with age in those who attain significant age. Self-selection into this group implies a different physiological handling of fetuin A. Understanding how and why this occurs could be quite illuminating.

4 Sirtuin Protein Expression in Normal Colorectal Tissue and in Colorectal Tumour Tissue and Relationship with Survival in Colorectal Cancer

4.1 Introduction

The yeast Sir2 proteins came to attention because of their effect on longevity of yeast cells (Imai, Armstrong et al. 2000). Sirtuins are human homologues of yeast Sir2 proteins; there are 7 sirtuins, SIRT1-7, and they have diverse effects on processes from metabolic regulation and cell-cycle control to DNA repair(Dali-Youcef, Lagouge et al. 2007). Polymorphisms in SIRT3 have been shown to associate with human longevity (Rose, Dato et al. 2003).

Altered RNA expression of sirtuins in human breast (Ashraf, Zino et al. 2006) and colorectal cancer (F Maxwell, unpublished data) was demonstrated in this laboratory. A Pubmed search was conducted on 4 Nov 2012, using the MeSH terms Sirtuins and Immunohistochemistry indicated that there were no published immunohistochemical studies defining the subcellular localisation of the 7 human sirtuins in the large intestine and linking protein expression with disease outcomes.

Protein levels of sirtuins 2-7 were individually assessed on a human colorectal TMA. Data from these investigations are presented below. Descriptive statistics, graphical representation and photographic examples of staining patterns for each sirtuin, in nuclei and cytoplasm, in normal and tumour tissue are detailed. Sirtuin protein expression in normal and tumour tissue was compared.

Associations between survival and Histoscores for each combination of individual sirtuin and subcellular location in tumour samples were examined and data on univariate and multivariate analysis is presented. There were insufficient numbers of normal samples for meaningful analysis of relationship with survival.

4.2 SIRT2 Immunohistochemistry

Representative pictures of sections stained for SIRT2 are shown.

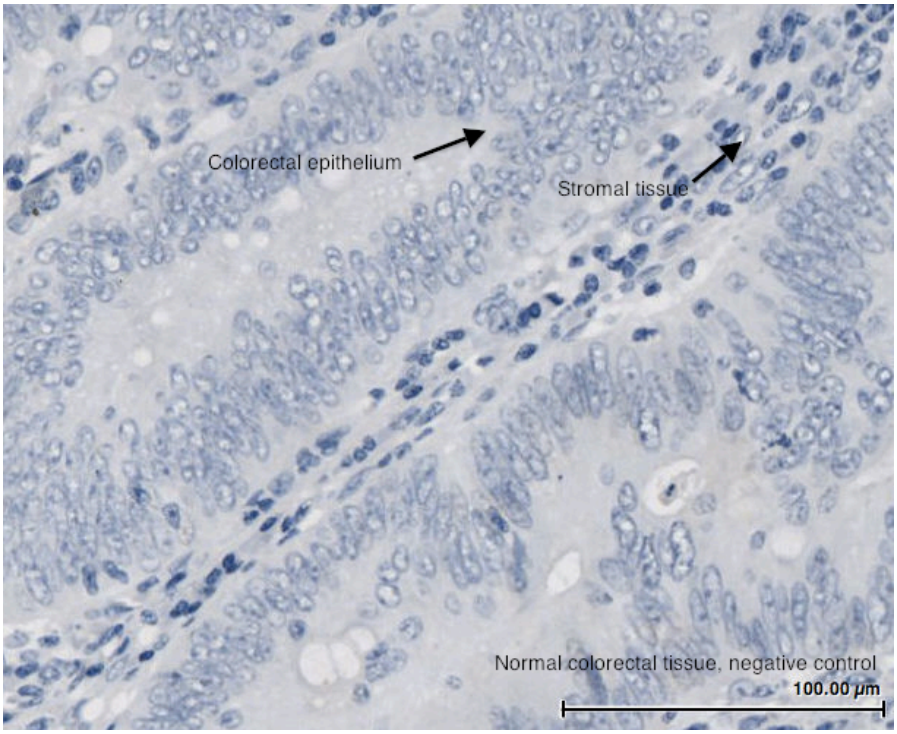

Figure 4.1 Normal colorectal tissue. Negative control stained for SIRT2.

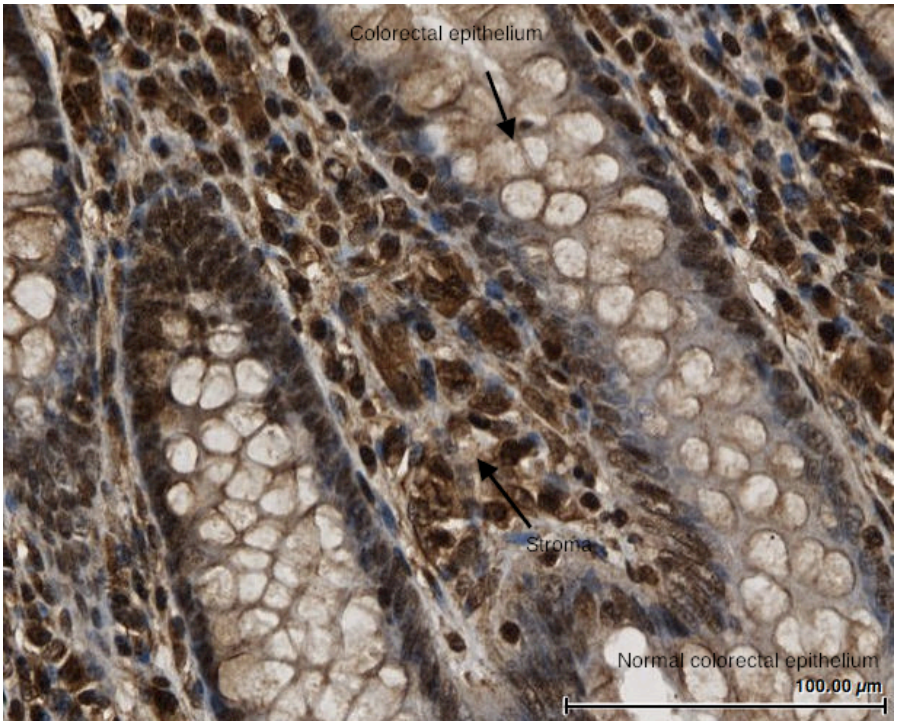

Figure 4.2 Normal colorectal tissue stained for SIRT2. Arrows show normal colorectal epithelium and stromal tissue.

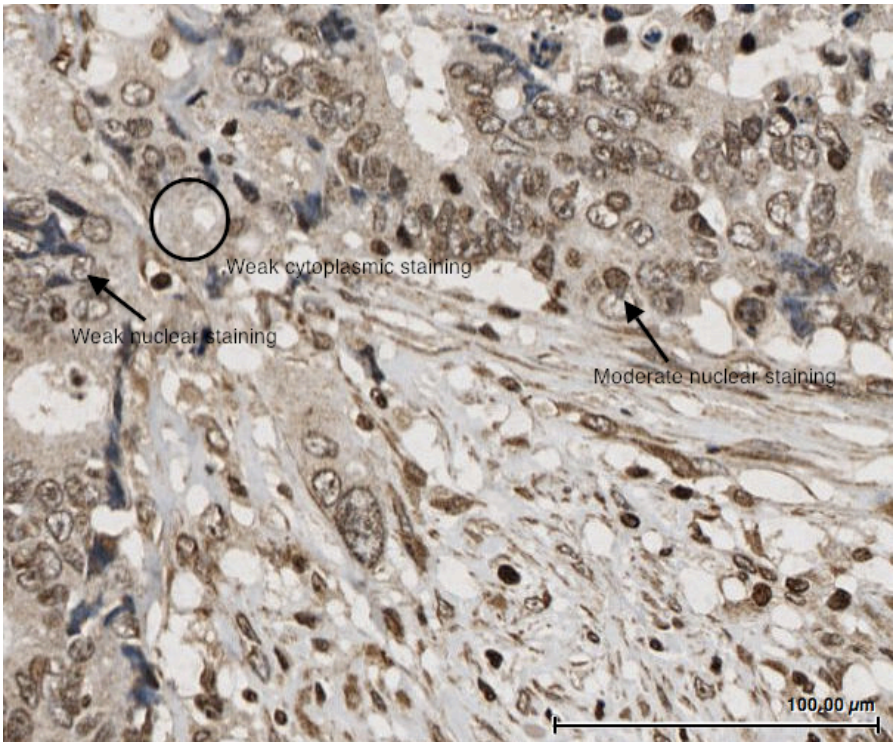

Figure 4.3 Colorectal tumour tissue stained for SIRT2. Arrows show weak and moderate nuclear staining, and generalised weak cytoplasmic staining, some of which is highlighted by the circle.

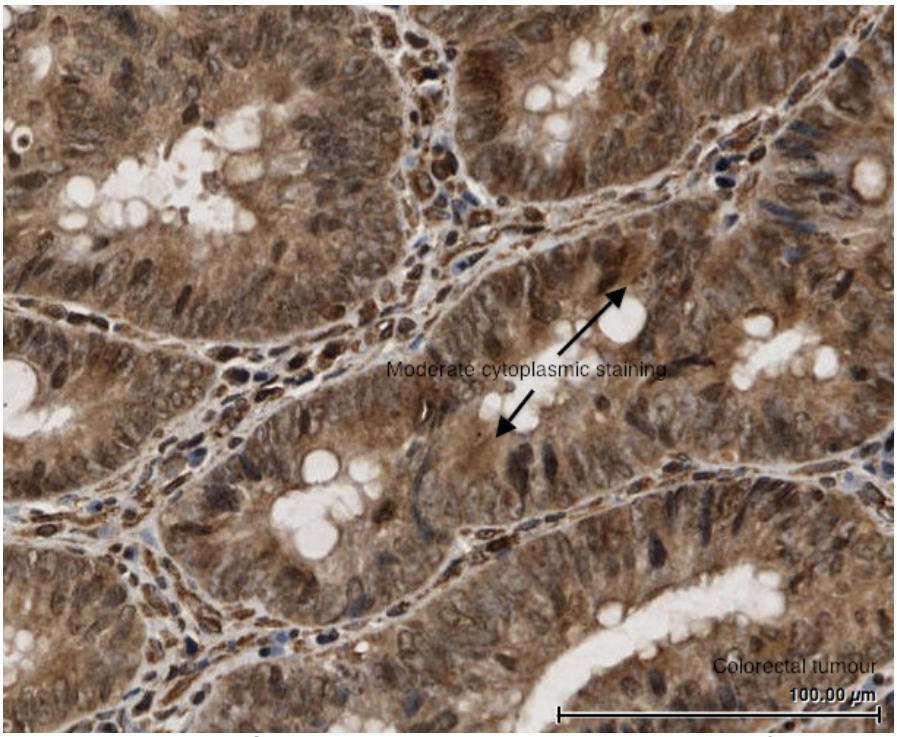

Figure 4.4 Variable SIRT2 cytoplasmic staining, with areas of moderate staining indicated by arrows. These were representative of the strongest staining areas of cytoplasm.

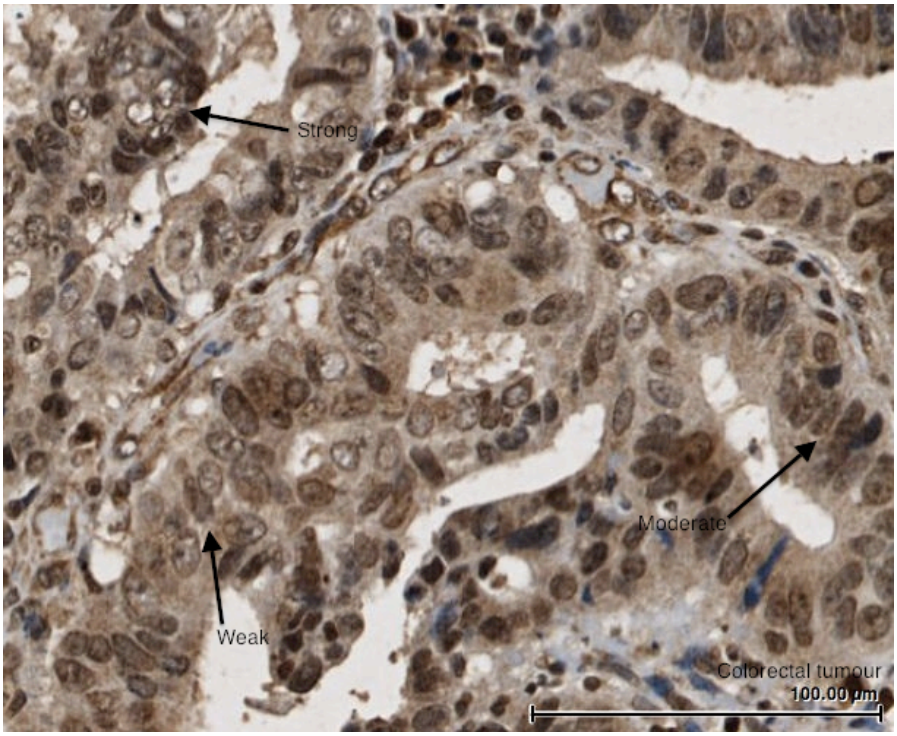

Figure 4.5 Variable SIRT2 nuclear staining with arrows indicating weak, moderate and strong staining.

Basic descriptive statistics for SIRT2 staining are given below.

SIRT2 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**165.00**	**90.00**	**97.50**	**290.00**	**135.00**	**225.00**
Tumour tissue	**105.00**	**48.75**	**0.00**	**218.33**	**76.25**	**125.00**
SIRT2 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**130.00**	**33.75**	**60.00**	**190.00**	**120.00**	**150.00**
Tumour tissue	**111.25**	**35.00**	**48.75**	**196.25**	**97.50**	**132.50**

Table 4.1 Descriptive statistics for SIRT2 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

4.2.1 SIRT2 protein expression levels in the nucleus in normal and tumour tissue.

Distribution of SIRT2 Histoscores in nuclei is represented graphically below for both normal and tumour tissue.

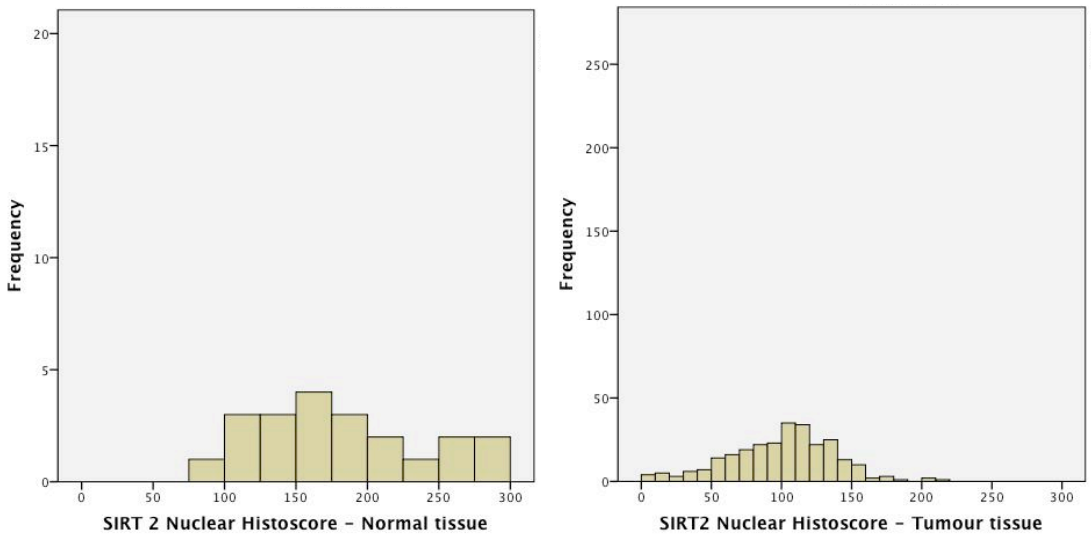

Figure 4.6 Two histograms showing the frequency distribution of SIRT2 Histoscores in nuclei, in normal and tumour tissue samples.

Staining intensity was generally less in tumour than normal tissue and this was confirmed on related samples Wilcoxon Signed Rank test (p=0.00008). Thus matched levels of SIRT2 protein in the nucleus were significantly lower in colorectal tumour cells than in normal colorectal cells.

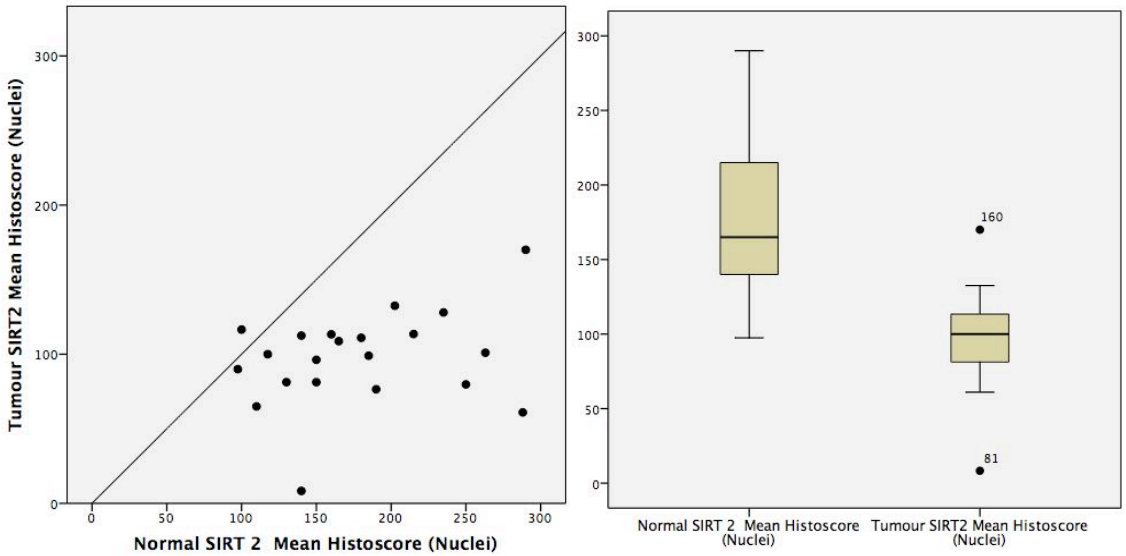

Figure 4.7 Scatterplot shows SIRT2 nuclear Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in normal than in tumour tissue. The boxplots of SIRT2 nuclear Histoscores from matched samples of colorectal tumour and normal tissue showed lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.00008).

4.2.2 SIRT2 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT2 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue.

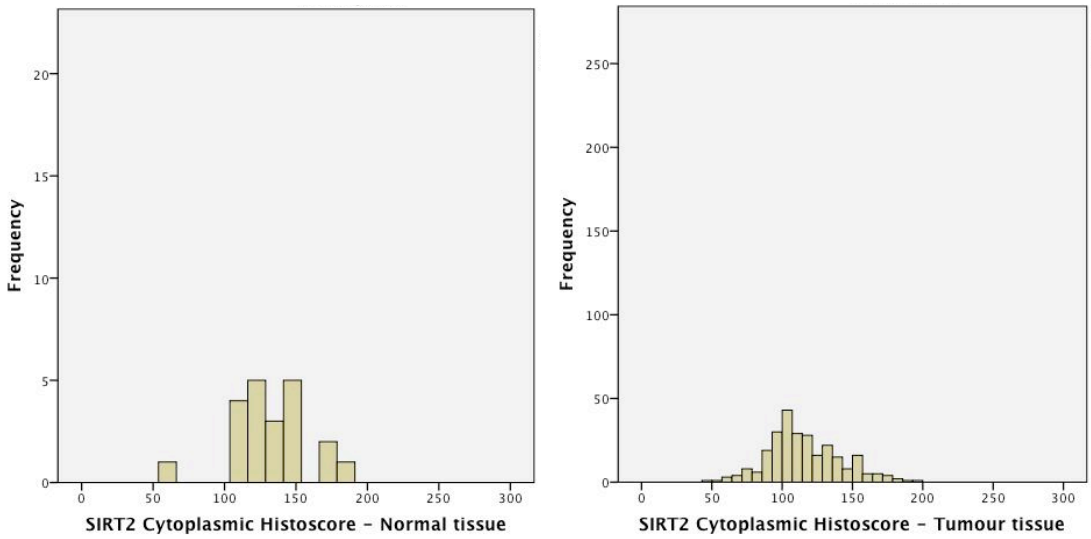

Figure 4.8 Two histograms showing the frequency distribution of SIRT2 Histoscores in cytoplasm, in normal and tumour tissue samples.

Staining intensity was generally less in tumour than normal tissue and this was confirmed on related samples Wilcoxon Signed Rank test (p=0.0082). Thus matched levels of SIRT2 protein in the cytoplasm were significantly lower in colorectal tumour cells than in normal colorectal cells.

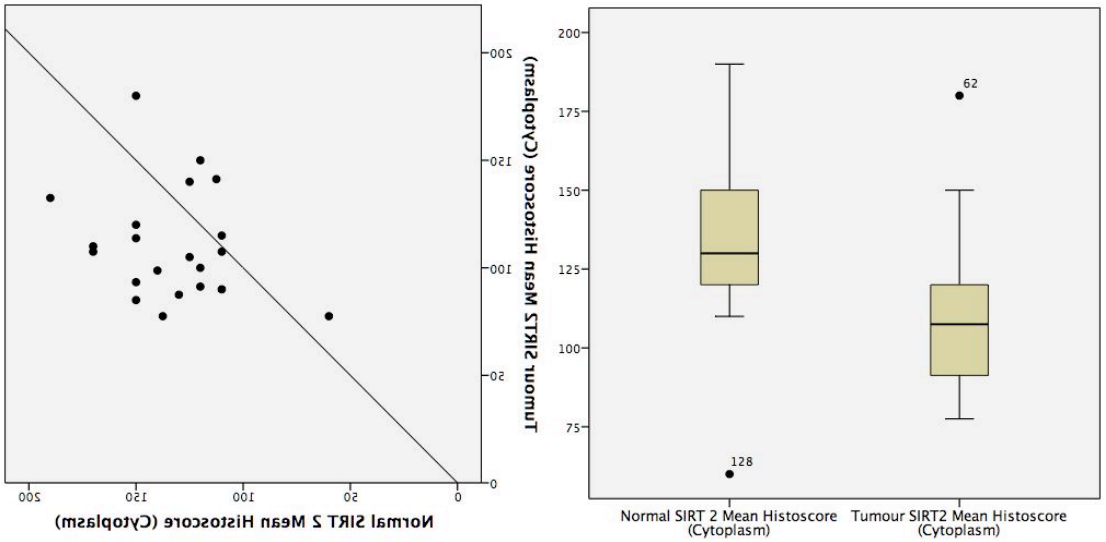

Figure 4.9 Scatterplot shows SIRT2 cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in normal than in tumour tissue. The boxplots of SIRT2 cytoplasmic Histoscores from matched samples of colorectal tumour and normal tissue showed lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.0082).

4.3 SIRT3 Immunohistochemistry

Representative pictures of sections stained for SIRT3 are shown. Staining appears granular when it is stronger.

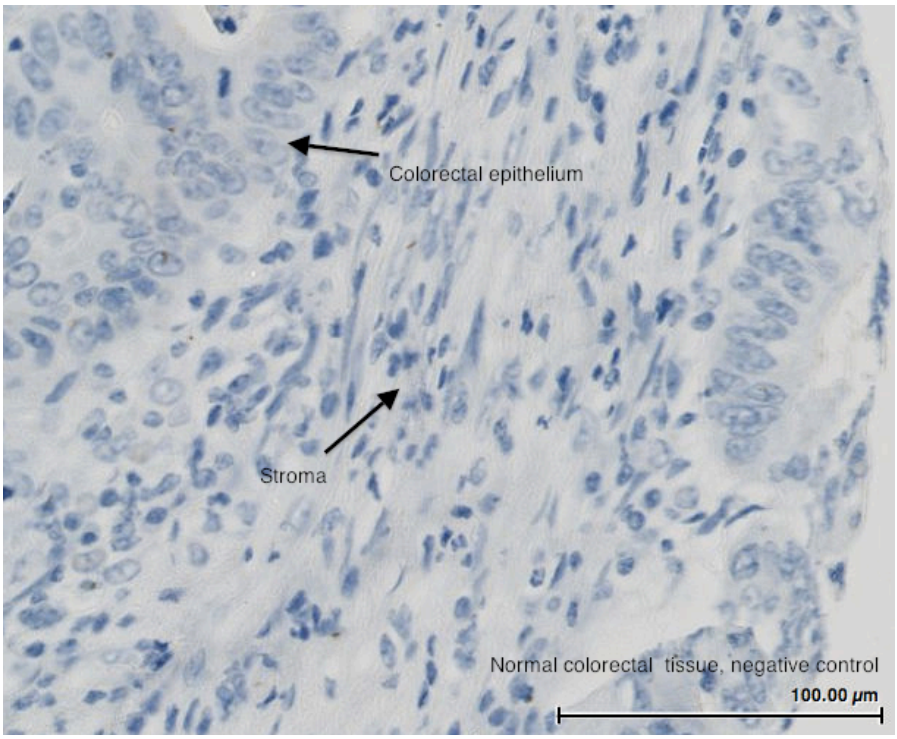

Figure 4.10 Normal colorectal tissue, negative control. Arrows indicate colorectal epithelium and stroma.

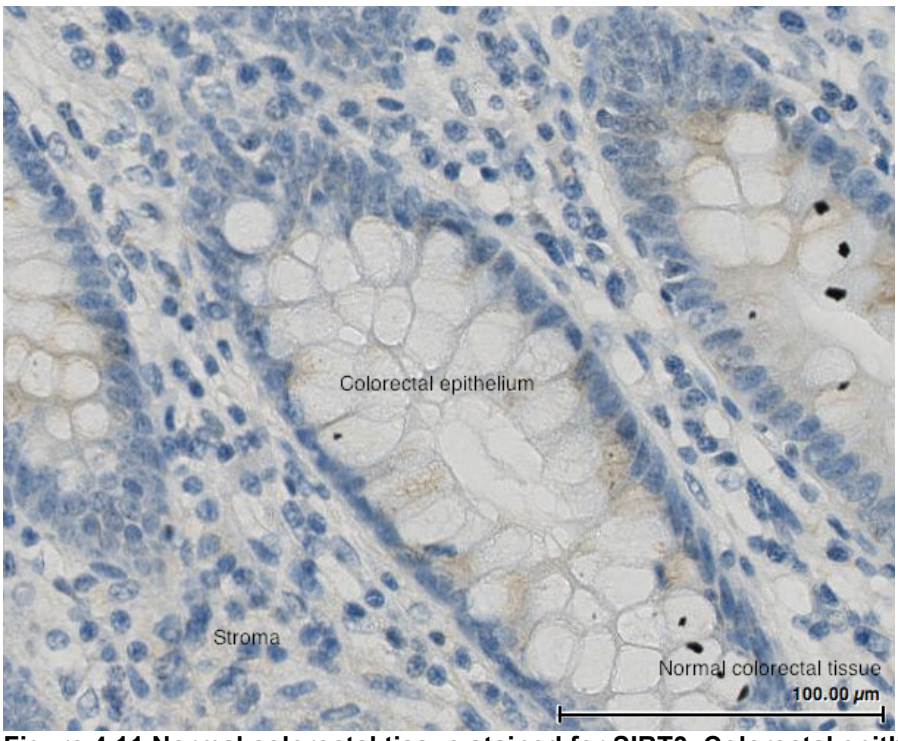

Figure 4.11 Normal colorectal tissue stained for SIRT3. Colorectal epithelium and stroma are labelled.

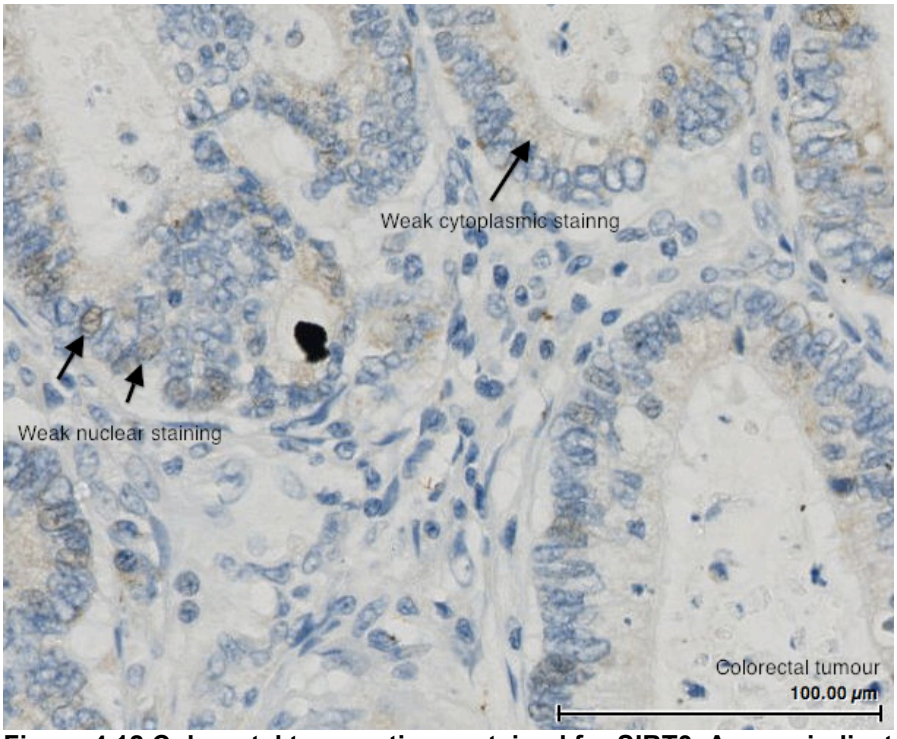

Figure 4.12 Colorectal tumour tissue stained for SIRT3. Arrows indicate weak nuclear and cytoplasmic staining.

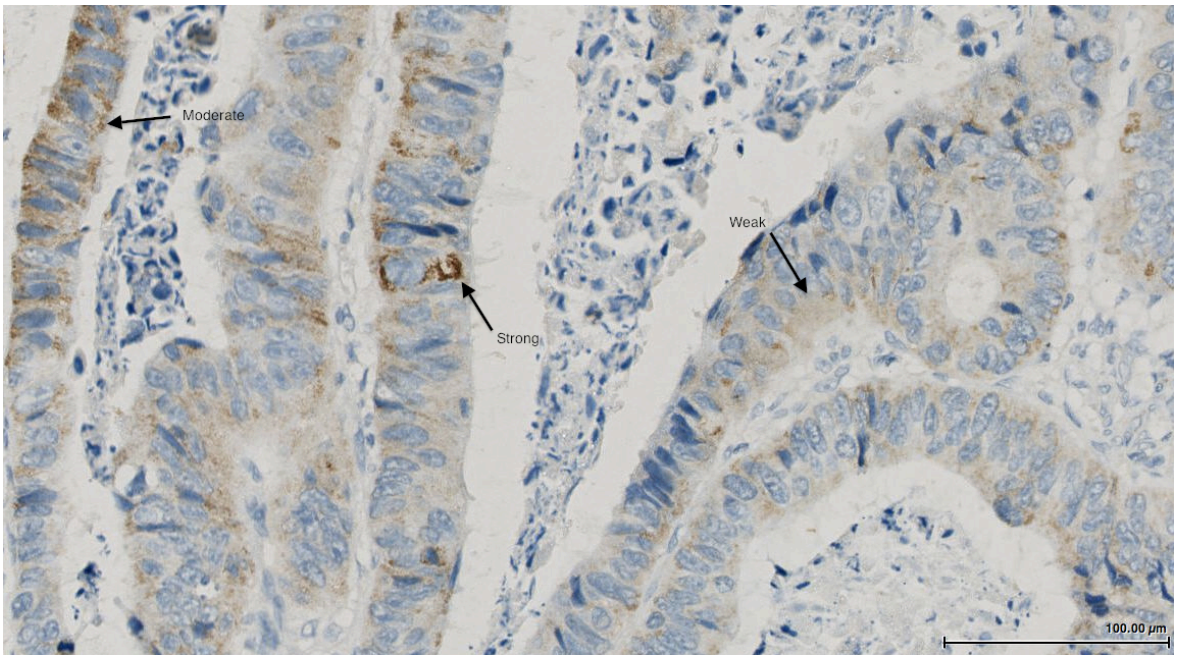

Figure 4.13 Cytoplasmic staining in colorectal tumour tissue stained for SIRT3. Arrows indicate weak, moderate and strong staining.

Basic descriptive statistics for SIRT3 staining are given below.

SIRT3 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**0.00**	**1.00**	**0.00**	**3.00**	**0.00**	**1.00**
Tumour tissue	**0.50**	**1.50**	**0.00**	**12.00**	**0.00**	**1.50**
SIRT3 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**2.00**	**4.38**	**0.00**	**40.00**	**1.00**	**5.00**
Tumour tissue	**17.50**	**34.25**	**0.00**	**181.25**	**5.00**	**39.25**

Table 4.2 Descriptive statistics for SIRT3 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

Staining intensity was overall very low, particularly in the nucleus; however, it appeared that SIRT3 protein is located in both the nucleus and the cytoplasm in colorectal tissue. As the antibody used here does not distinguish between the short and long isoforms of SIRT3, it is not possible to determine if the isoforms are differentially localised. SIRT3 protein expression levels in the nucleus in normal and tumour tissue.

4.3.1 SIRT3 protein expression levels in nuclei in normal and tumour tissue.

Distribution of SIRT3 Histoscores in nuclei is represented graphically below for both normal and tumour tissue.

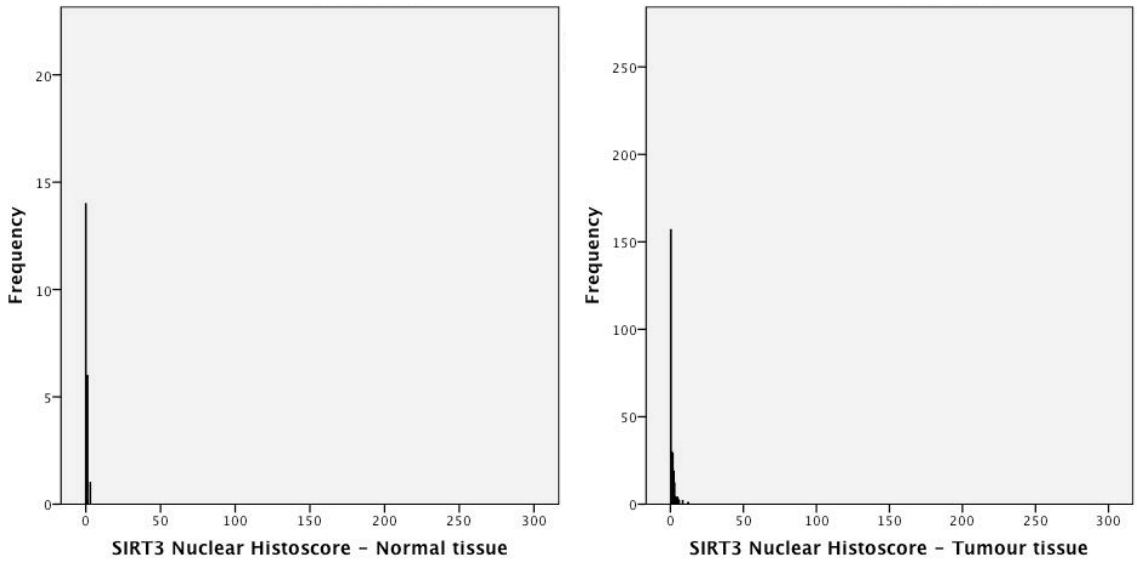

Figure 4.14 Two histograms show the frequency distribution of SIRT3 Histoscores in nuclei, in normal and tumour tissue samples.

Related samples Wilcoxon Signed Rank test comparing the distribution of Histoscores in both tissues confirmed there was no significant difference in staining (p=0.3915).

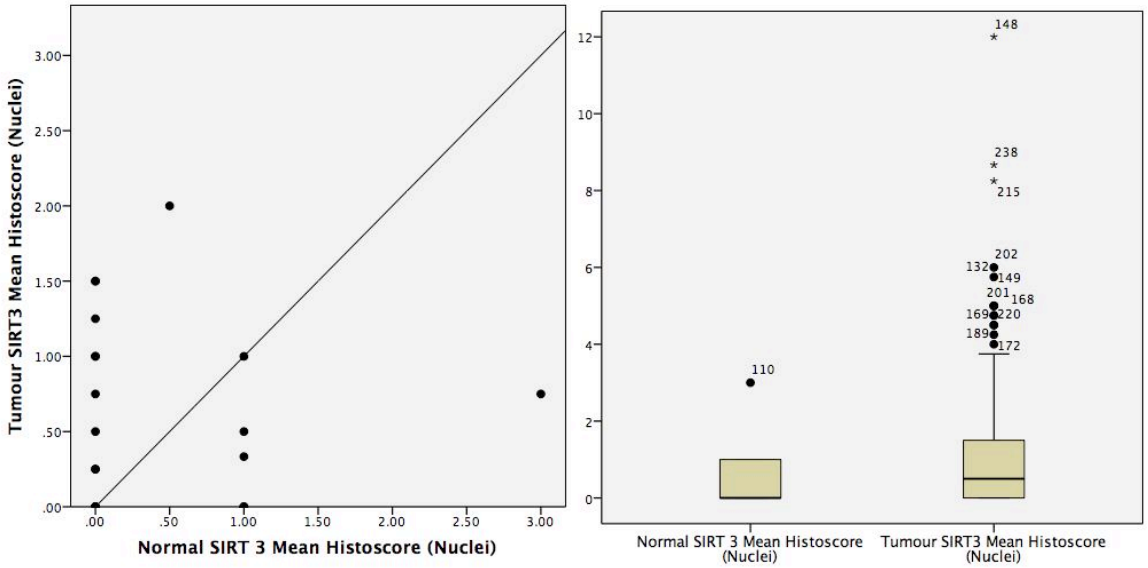

Figure 4.15 SIRT3 nuclear Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue are plotted. The solid line is the line of equivalence. There was no significant difference in staining in matched normal and tumour tissue (Wilcoxon Signed Ranks test, p=0.3915). Boxplot of SIRT3 nuclear Histoscores from the full cohort of normal and colorectal tumour tissue samples, Median Histoscore is higher in tumour than in normal tissue (Mann-Whitney U test, p=0.029).

Analysis of the full cohort as unmatched samples confirmed that staining in tumour tissue is higher than in normal tissue (Mann-Whitney U test, p=0.029). Thus levels of SIRT3 protein in nuclei were significantly higher in colorectal tumour cells than in normal colorectal cells.

4.3.2 SIRT3 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT3 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue.

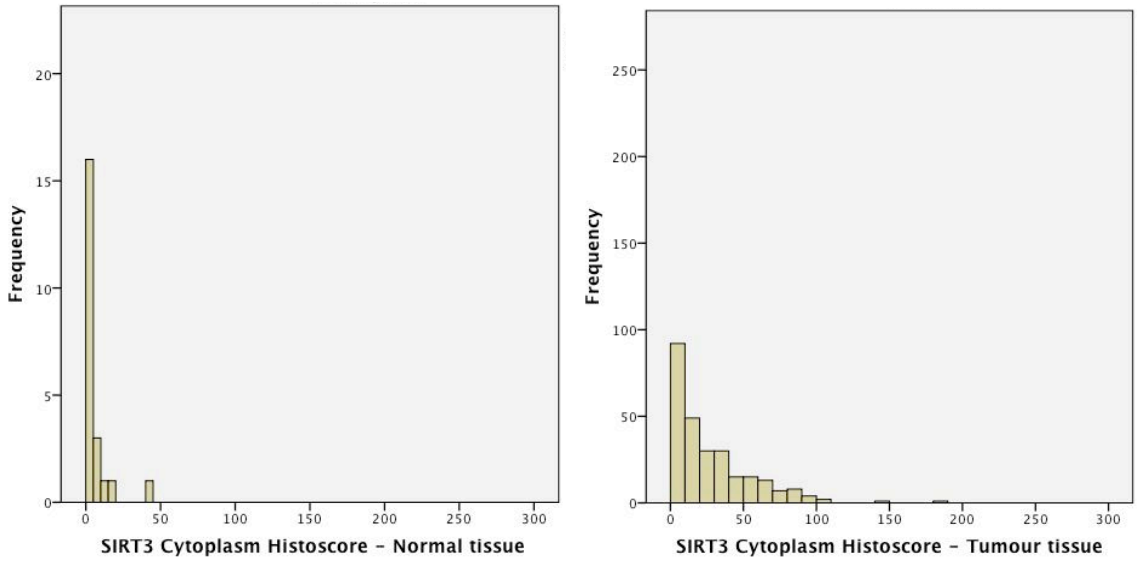

Figure 4.16 Two histograms show the frequency distribution of SIRT3 Histoscores in cytoplasm, in normal and tumour tissue samples.

Wilcoxon Signed Rank test comparing the distribution of Histoscores in both tissues confirmed staining was significantly stronger in tumour tissue (p=0.0015). Thus matched levels of SIRT3 protein in the cytoplasm were significantly lower in colorectal tumour cells than in normal colorectal cells.

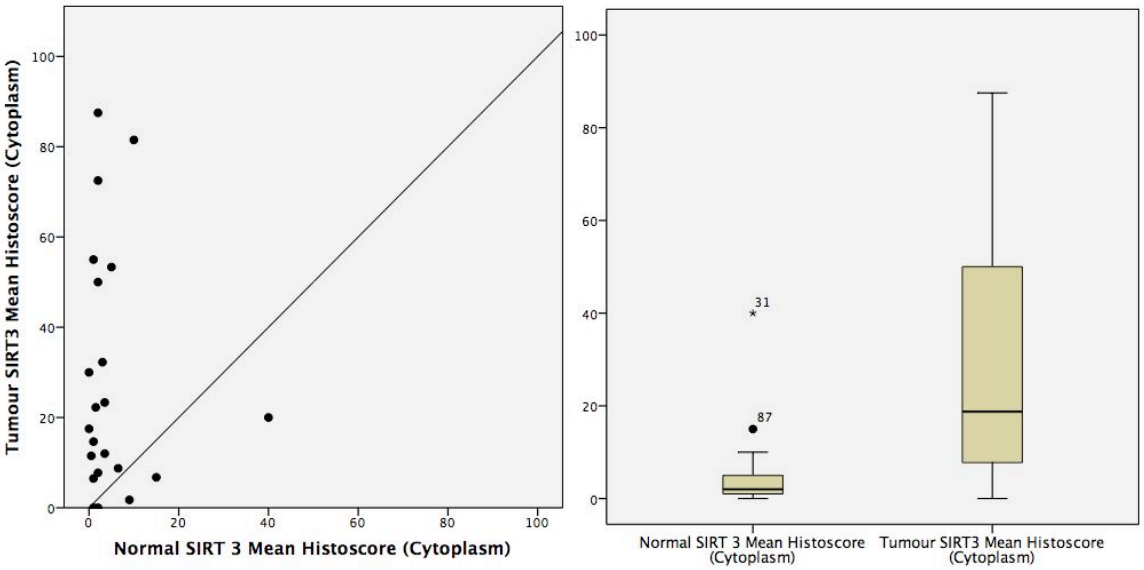

Figure 4.17 The scatterplot shows SIRT3 cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in tumour than in normal tissue. The boxplots show SIRT3 cytoplasmic Histoscores from matched samples of normal and colorectal tumour tissue, with higher Histoscores in tumour samples. This difference is statistically significant (Wilcoxon Signed Ranks test, p=0.0015).

Thus SIRT3 protein levels in the cytoplasm were higher in tumour tissue.

4.4 SIRT4 Immunohistochemistry

Representative pictures of sections stained for SIRT4 are shown.

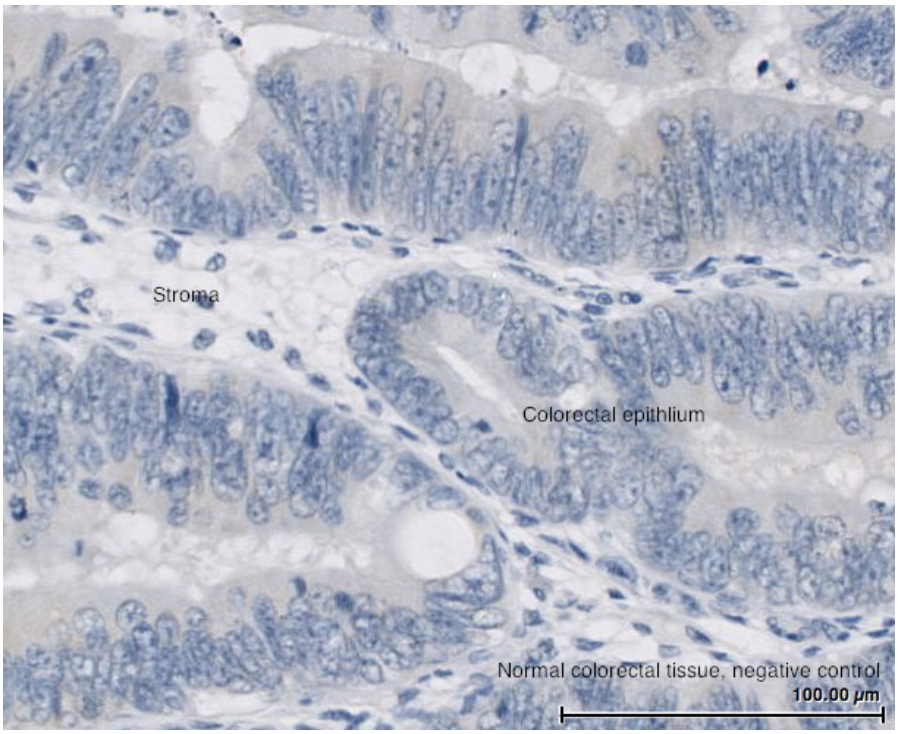

Figure 4.18 Normal colorectal tissue, negative control. Colorectal epithelium and stroma are labelled.

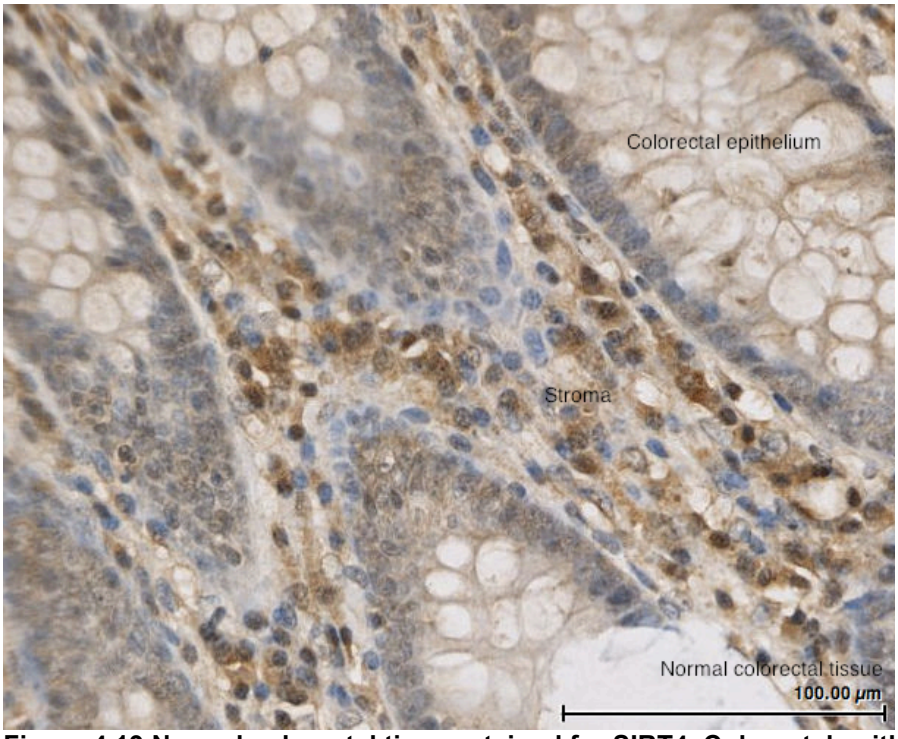

Figure 4.19 Normal colorectal tissue stained for SIRT4. Colorectal epithelium and stroma are labelled.

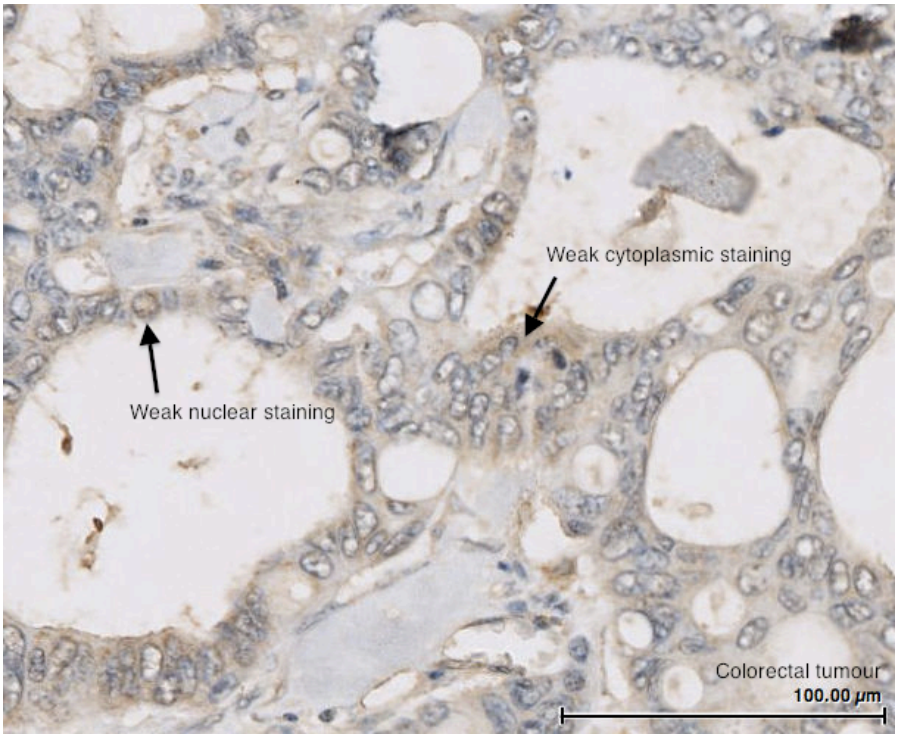

Figure 4.20 Colorectal tumour tissue stained for SIRT4. Arrows show weak nuclear and cytoplasmic staining.

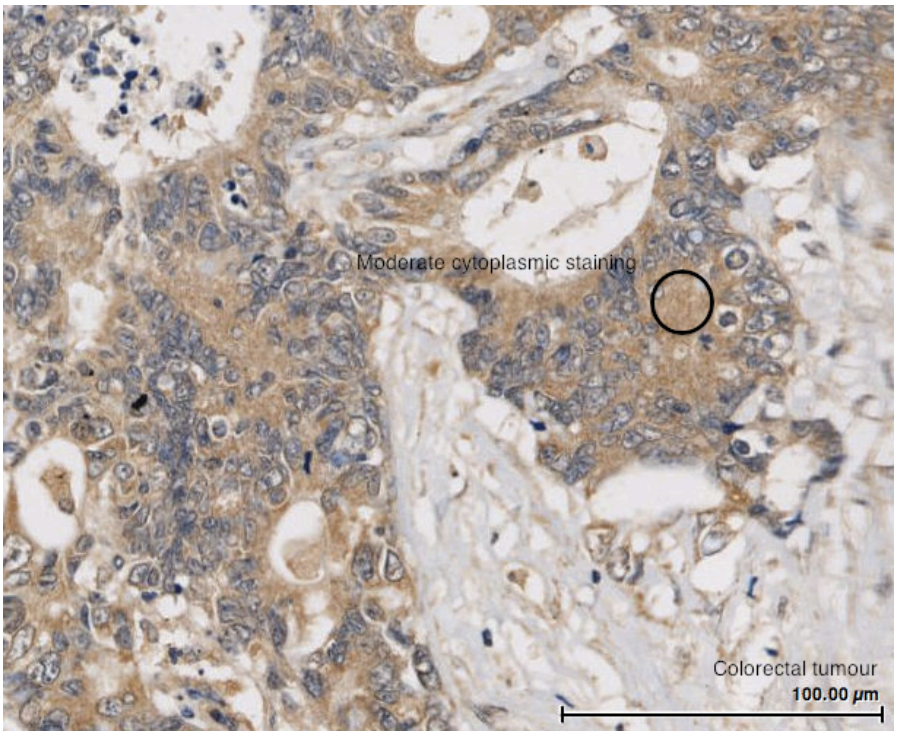

Figure 4.21 Colorectal tumour tissue stained for SIRT4. This section shows generally moderate cytoplasmic staining, highlighted by the circle. Nuclear staining did not reach moderate levels in tumour tissue.

Basic descriptive statistics for SIRT4 staining are given below.

SIRT4 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**12.25**	**16.88**	**0.00**	**80.00**	**5.00**	**20.00**
Tumour tissue	**0.00**	**1.25**	**0.00**	**16.25**	**0.00**	**1.25**
SIRT4 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**65.00**	**46.25**	**0.00**	**125.00**	**40.00**	**80.00**
Tumour tissue	**50.00**	**42.81**	**1.25**	**122.50**	**27.19**	**70.00**

Table 4.3 Descriptive statistics for SIRT4 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

As one of the 'mitochondrial sirtuins', nuclear staining was unexpected for SIRT4 and there are no previous reports of this, but the intensity of staining for SIRT4 in the nucleus was higher than staining for SIRT3, for which there is a reported precedent. Staining intensity was lower in nuclei than in cytoplasm.

4.4.1 SIRT4 protein expression levels in the nucleus in normal and tumour tissue.

Distribution of SIRT4 Histoscores in nuclei is represented graphically below for both normal and tumour tissue.

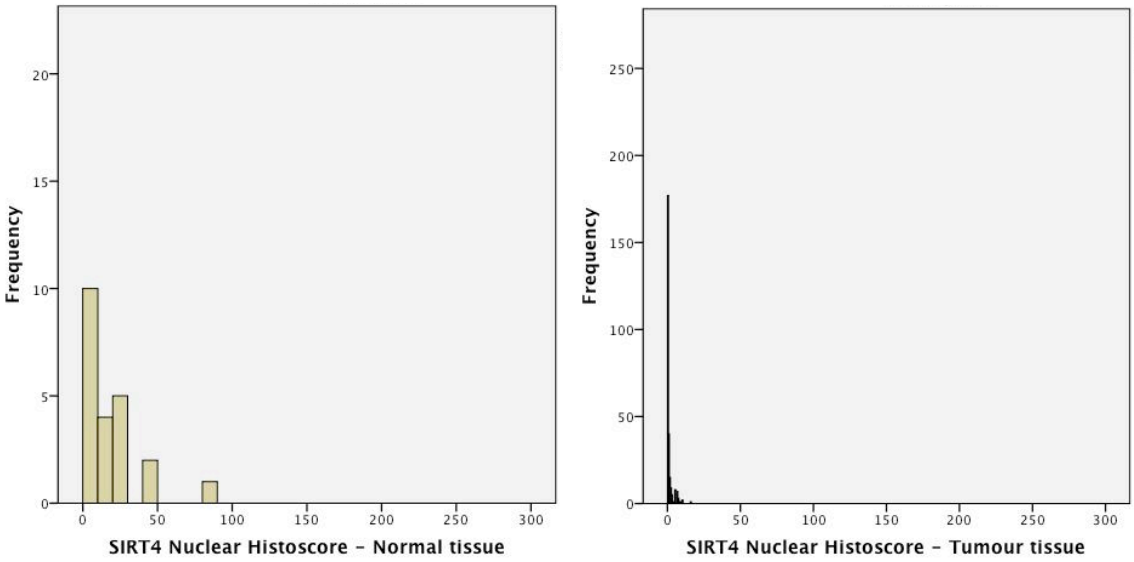

Figure 4.22 Two histograms showing the frequency distribution of SIRT4 Histoscores in nuclei, in normal and tumour tissue samples

Staining intensity was generally less in tumour than normal tissue and this was confirmed on related samples Wilcoxon Signed Rank test (p=0.00016). Thus matched levels of SIRT4 protein in nuclei were significantly lower in colorectal tumour cells than in normal colorectal cells.

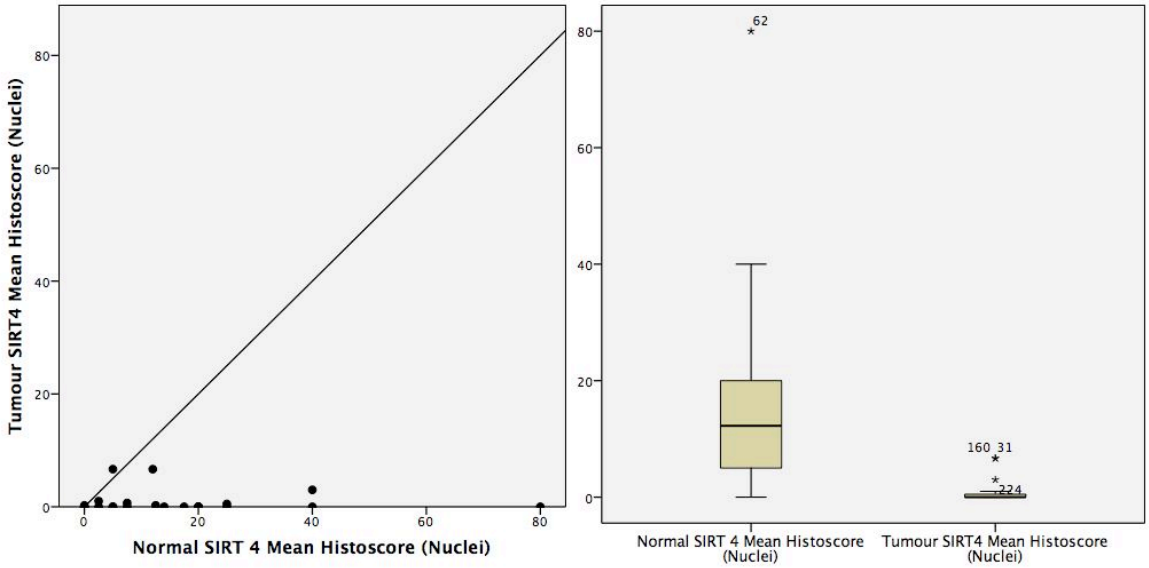

Figure 4.23 Scatterplot shows SIRT4 nuclear Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in normal than in tumour tissue. The boxplots of SIRT4 nuclear Histoscores from matched samples of colorectal tumour and normal tissue showed lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.00016).

4.4.2 SIRT4 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT4 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue.

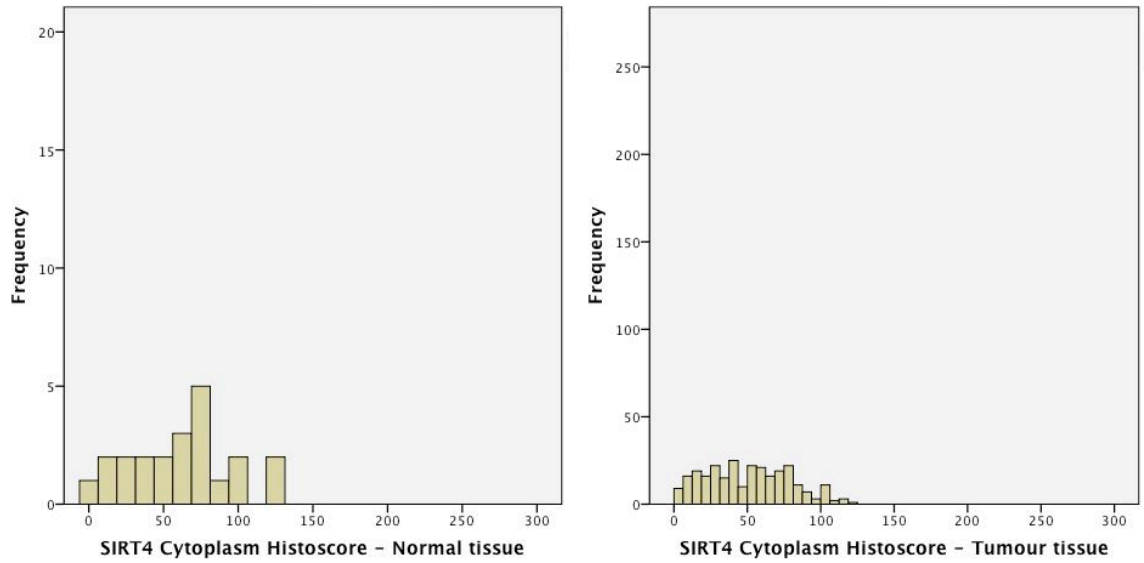

Figure 4.24 Two histograms show the frequency distribution of SIRT4 Histoscores in cytoplasm, in normal and tumour tissue samples.

There was a significant correlation between cytoplasmic Histoscores for both normal and tumour tissue (Spearman correlation coefficient 0.549, p=0.008) with lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.041).

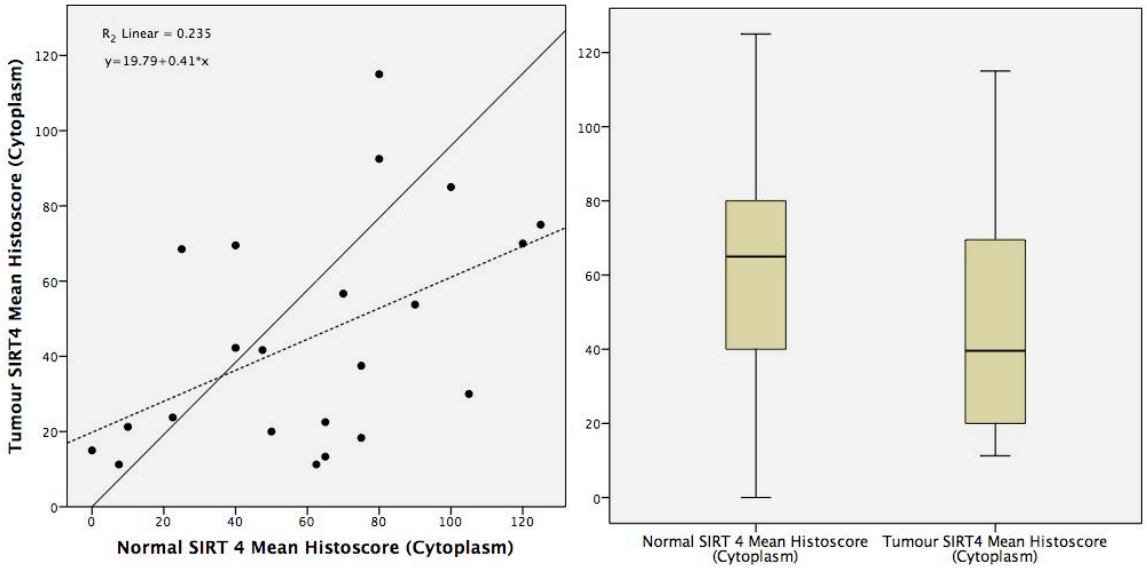

Figure 4.25 Scatterplot shows cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue stained for SIRT4. The solid line is the line of equivalence, the dashed line is the line of best fit (Spearman correlation coefficient 0.549, p=0.008). Most points showed lower Histoscore in tumour than in normal tissue. The boxplots of SIRT4 cytoplasmic Histoscores from matched samples of colorectal tumour and normal tissue showed lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.041).

SIRT4 protein levels in the cytoplasmic compartment were significantly lower in colorectal tumour tissue than in adjacent normal tissue.

4.5 SIRT5 Immunohistochemistry

Representative pictures of sections stained for SIRT5 are shown. There was no staining for SIRT5 in nuclei of any of the normal tissue samples available (n=20).

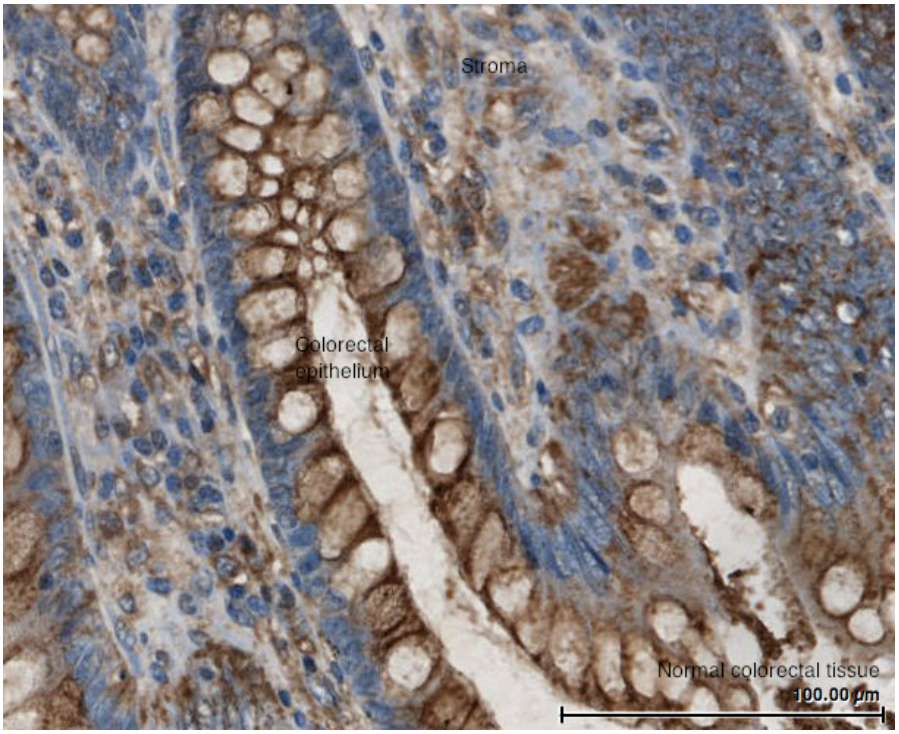

Figure 4.26 Normal colorectal tissue stained for SIRT5. Colorectal epithelium and stroma are labelled.

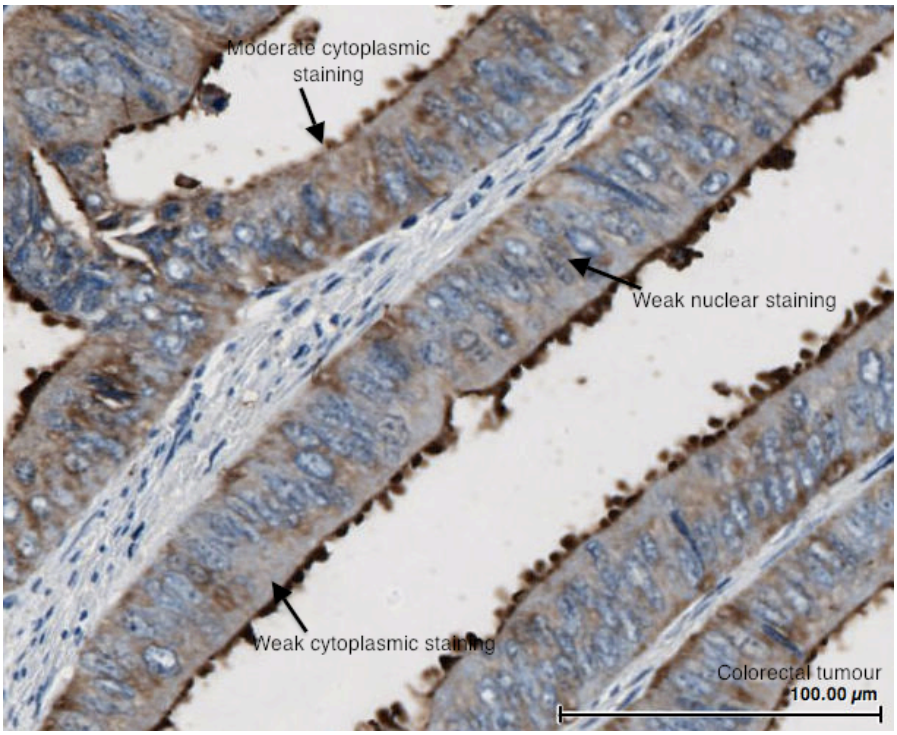

Figure 4.27 Colorectal tumour section stained for SIRT5. Arrows indicate few weakly stained nuclei. Cytoplasmic staining is patchy and of variable intensity within individual cells (arrows indicate areas of weak and moderate staining). There appears to be significant membrane-bound staining. Specific Histoscore was not calculated for cell membrane staining.

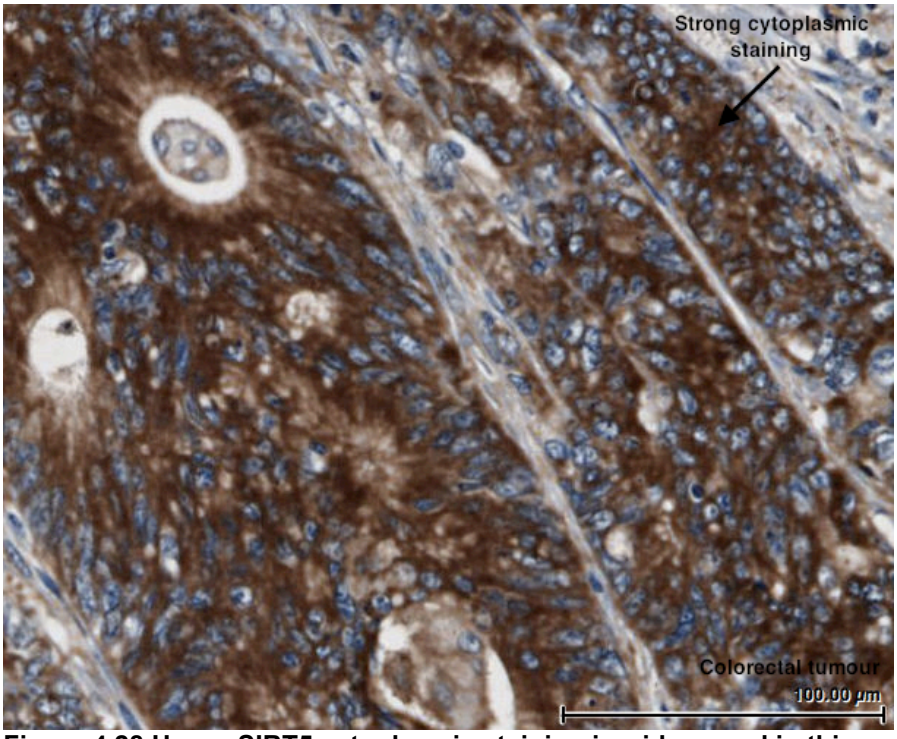

Figure 4.28 Heavy SIRT5 cytoplasmic staining is widespread in this section of colorectal tumour and is specifically indicated by the arrow.

Basic descriptive statistics for SIRT5 staining are given below.

SIRT5 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	*	*	*	*	*	*
Tumour tissue	**0.00**	**0.00**	**0.00**	**10.00**	**0.00**	**0.00**
SIRT5 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**152.50**	**52.50**	**85.00**	**220.00**	**137.50**	**187.50**
Tumour tissue	**88.54**	**52.19**	**2.50**	**230.00**	**67.50**	**119.69**

Table 4.4 Descriptive statistics for SIRT5 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

Staining intensity was overall very low, similar to SIRT3; staining was particularly low in the nucleus in tumour samples.

4.5.1 SIRT5 protein expression levels in the nucleus in normal and tumour tissue.

There was no staining for SIRT5 in the nucleus in normal tissue, so no comparison was possible.

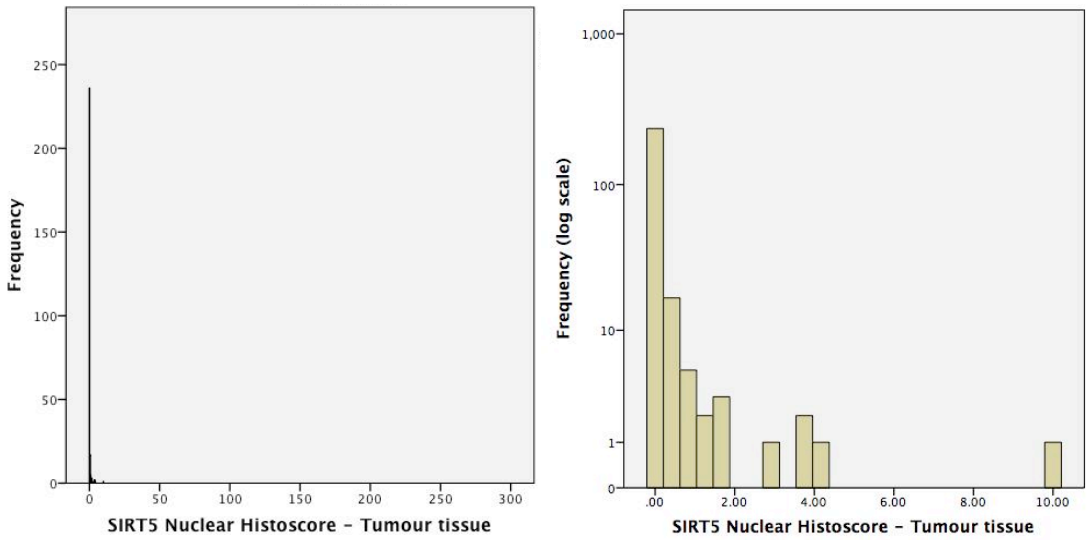

Figure 4.29 Distribution of SIRT5 Histoscores in nuclei is represented graphically below for tumour tissue, using both linear and logarithmic frequency scale.

As one of the 'mitochondrial sirtuins', nuclear staining was unexpected for SIRT5 as for SIRT4, and there are no previous reports of this. Histoscores were very low in tumour tissue and a larger study of normal tissue samples would be required to confirm nuclear expression of SIRT5 in normal tissue.

4.5.2 SIRT5 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT5 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue. Staining intensity was generally less in tumour than normal tissue and this was confirmed on related samples Wilcoxon Signed Rank test (p=0.00068). Thus matched levels of SIRT5 protein in the cytoplasm were significantly lower in colorectal tumour cells than in normal colorectal cells.

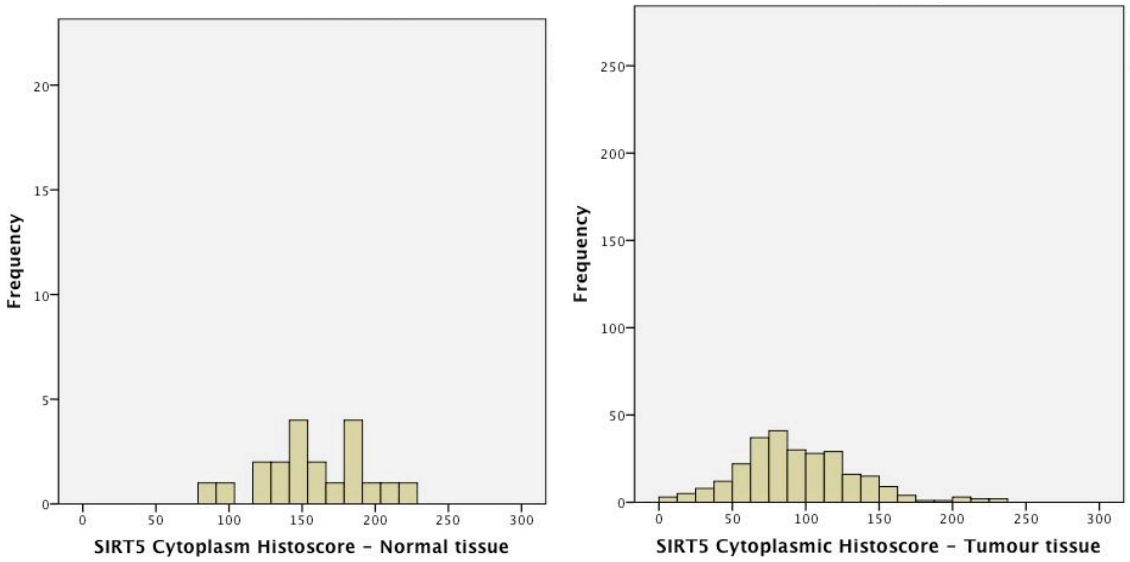

Figure 4.30 Two histograms show the frequency distribution of SIRT5 Histoscores in cytoplasm, in normal and tumour tissue samples.

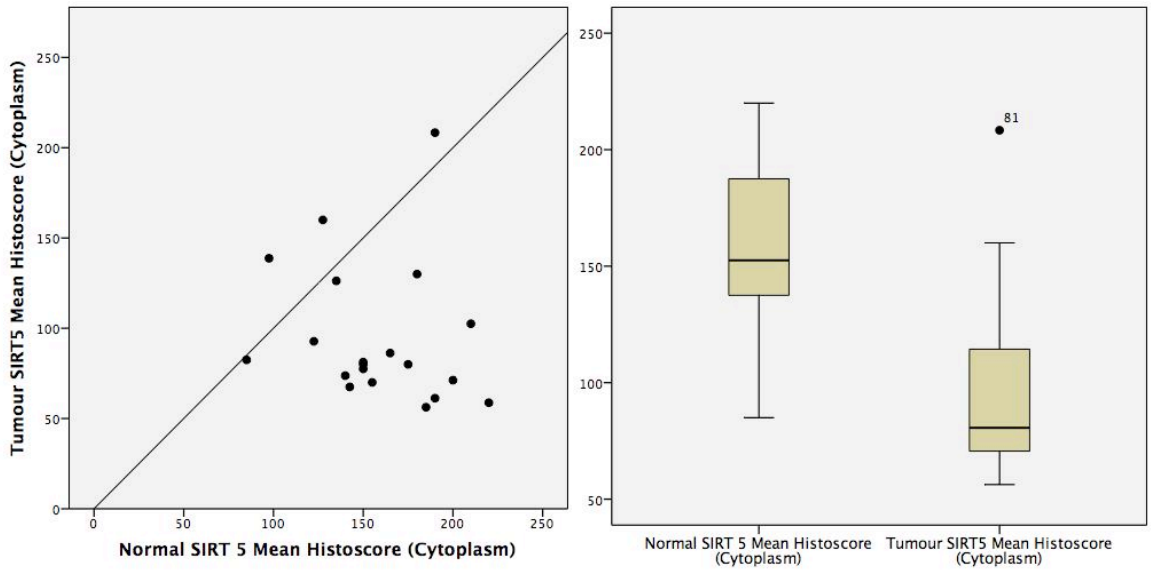

Figure 4.31 Scatterplot shows SIRT5 cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in normal than in tumour tissue. The boxplots of SIRT5 cytoplasmic Histoscores from matched samples of colorectal tumour and normal tissue showed lower Histoscores in tumour tissue (Wilcoxon Signed Rank test, p=0.00068).

4.6 SIRT6 Immunohistochemistry

Representative pictures of sections stained for SIRT6 are shown.

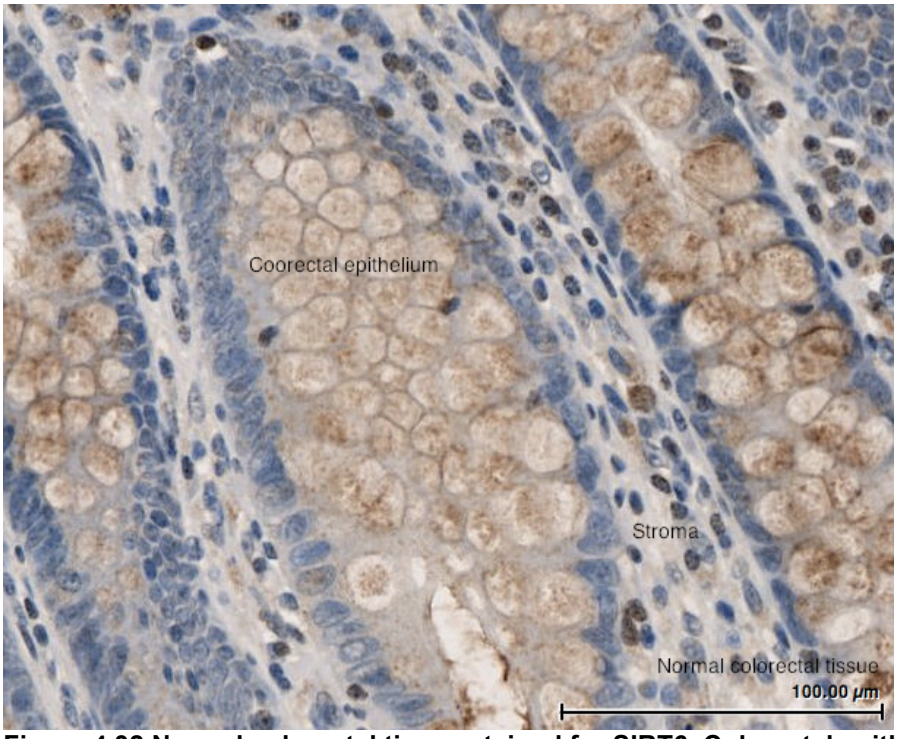

Figure 4.32 Normal colorectal tissue stained for SIRT6. Colorectal epithelium and stroma are labelled.

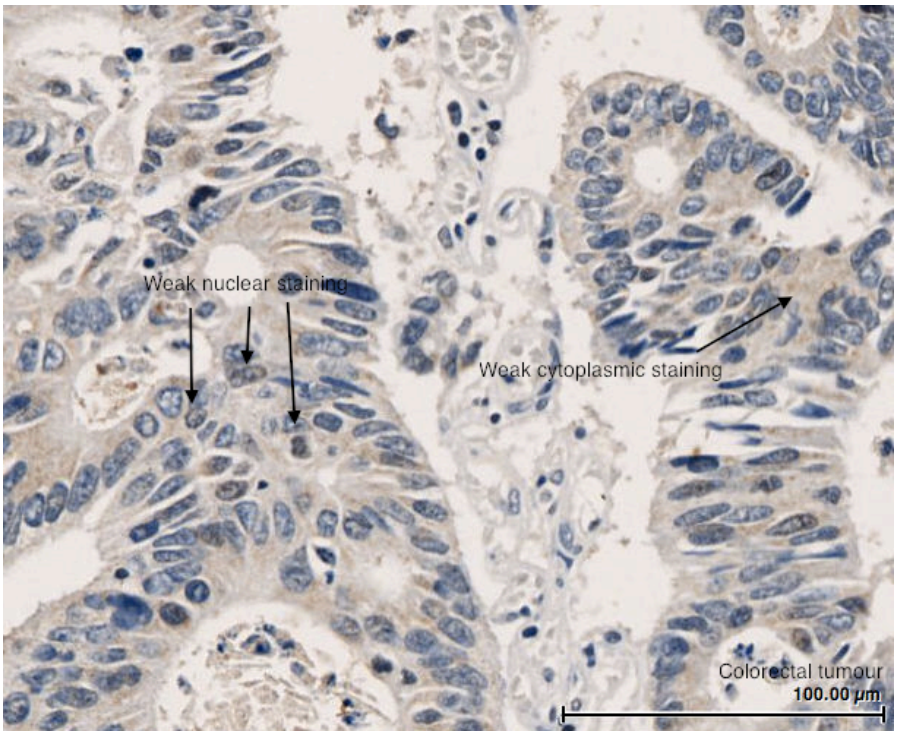

Figure 4.33 Colorectal tumour tissue stained for SIRT6. Cytoplasmic staining is generally weak (arrow highlights representative area). Nuclear staining is weak, where present (also indicated by arrows).

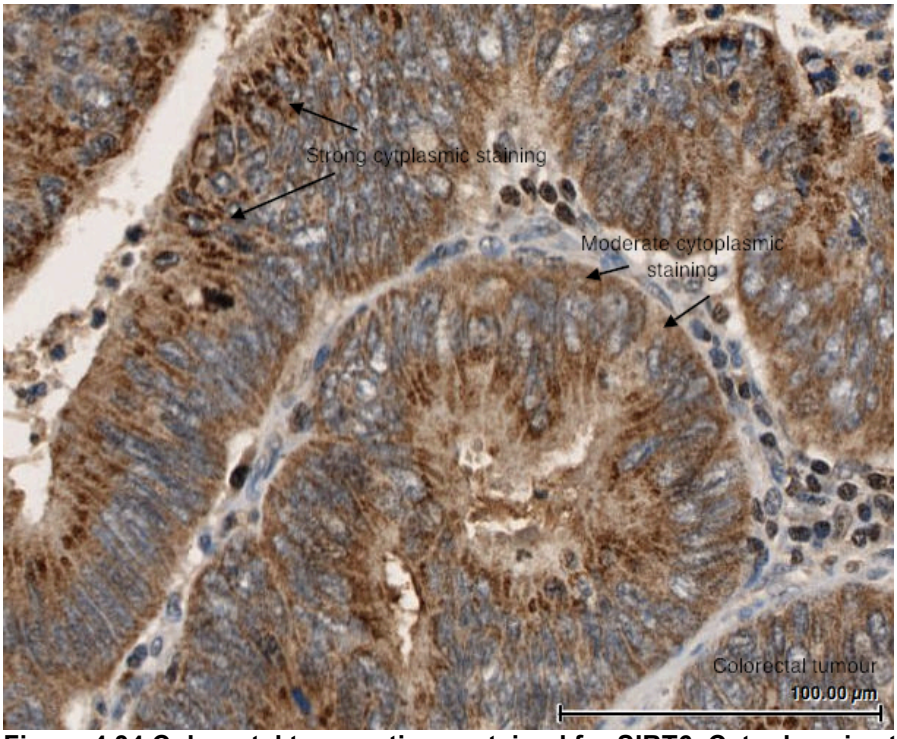

Figure 4.34 Colorectal tumour tissue stained for SIRT6. Cytoplasmic staining is variable within individual cells and appears stronger towards, but not close to the apical aspect. Arrows indicate contrasting areas of moderate and strong staining. Granular areas of strong staining may represent containment in intracellular organelles.

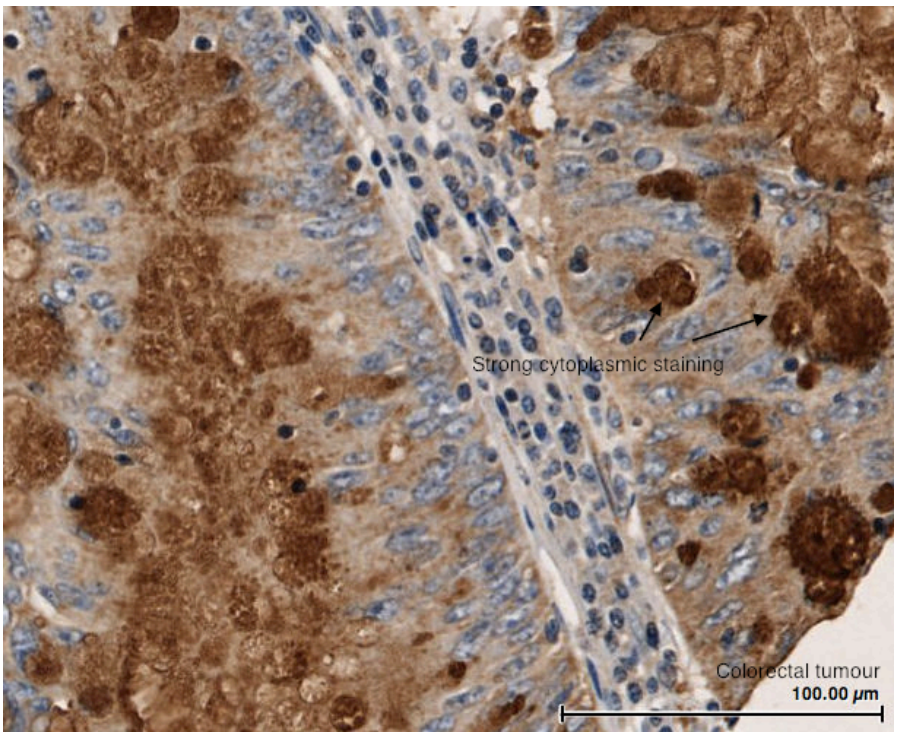

Figure 4.35 Colorectal tumour tissue stained for SIRT6. Strongest staining here appears to be at the apical aspect of the cell. Conglomerations of strongly staining tissue may indicate SIRT6 contained in intracellular organelles or vacuoles, possibly for secretion.

Basic descriptive statistics for SIRT6 staining are given below.

SIRT6 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**0.75**	**1.50**	**0.00**	**6.00**	**0.00**	**1.50**
Tumour tissue	**2.00**	**5.17**	**0.00**	**50.00**	**0.50**	**5.67**
SIRT6 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**69.50**	**35.63**	**34.00**	**140.00**	**55.00**	**88.75**
Tumour tissue	**90.00**	**57.25**	**0.00**	**210.00**	**62.50**	**119.75**

Table 4.5 Descriptive statistics for SIRT6 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

4.6.1 SIRT6 protein expression levels in the nucleus in normal and tumour tissue.

Distribution of SIRT6 Histoscores in nuclei is represented graphically below for both normal and tumour tissue.

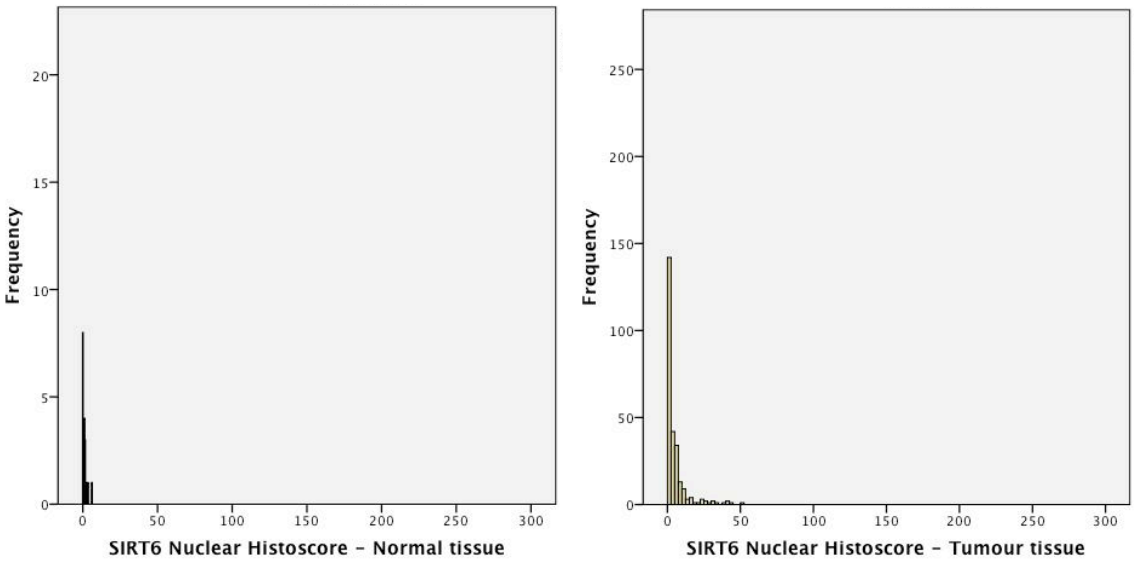

Figure 4.36 Two histograms showing the frequency distribution of SIRT6 Histoscores in nuclei, in normal and tumour tissue samples.

Wilcoxon Signed Rank test (p=0.011) comparing the distribution of Histoscores in both tissues, showed a significant difference in staining on exclusion of an outlier (sample 31). Comparison of the distribution of Histoscores in the full cohort of tumour samples showed the same pattern, with a more pronounced result (Mann-Whitney U test, p=0.0026).

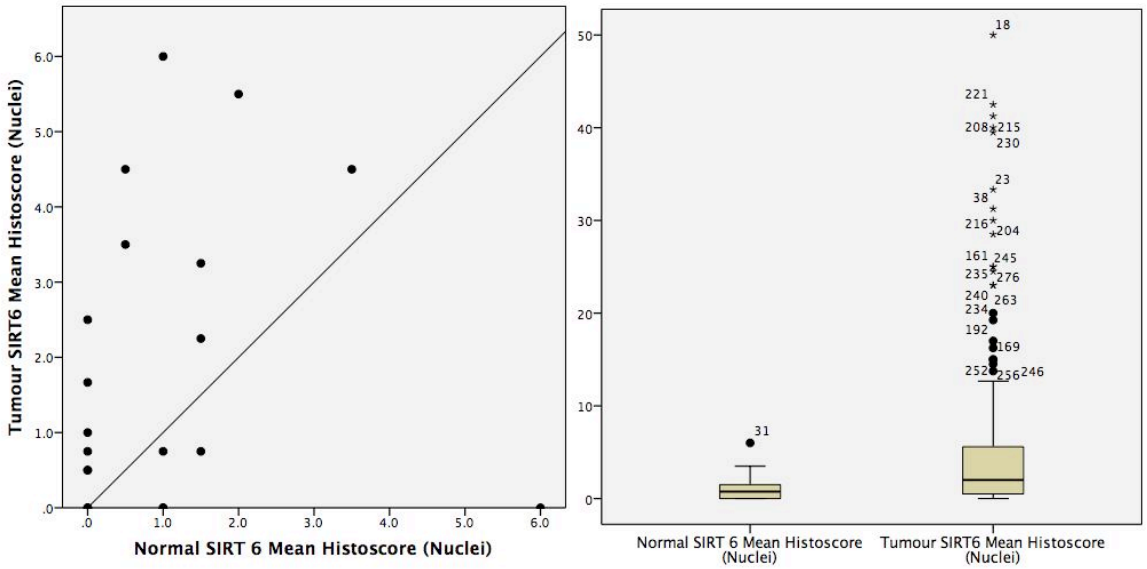

Figure 4.37 Nuclear Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue stained for SIRT6 are displayed on the scatterplot. The solid line is the line of equivalence. There was a statistically significant difference between nuclear Histoscores in normal and tumour tissue when the sample with high nuclear Histoscore in normal tissue was excluded (Wilcoxon Signed Ranks test, p=0.011). The boxplots of nuclear Histoscores from the full cohort of colorectal tumour and normal tissue samples showed higher Histoscores in tumour tissue (Mann-Whitney U test, p=0.0026).

Thus levels of SIRT6 protein in nuclei were significantly higher in colorectal tumour cells than in normal colorectal cells.

4.6.2 SIRT6 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT6 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue.

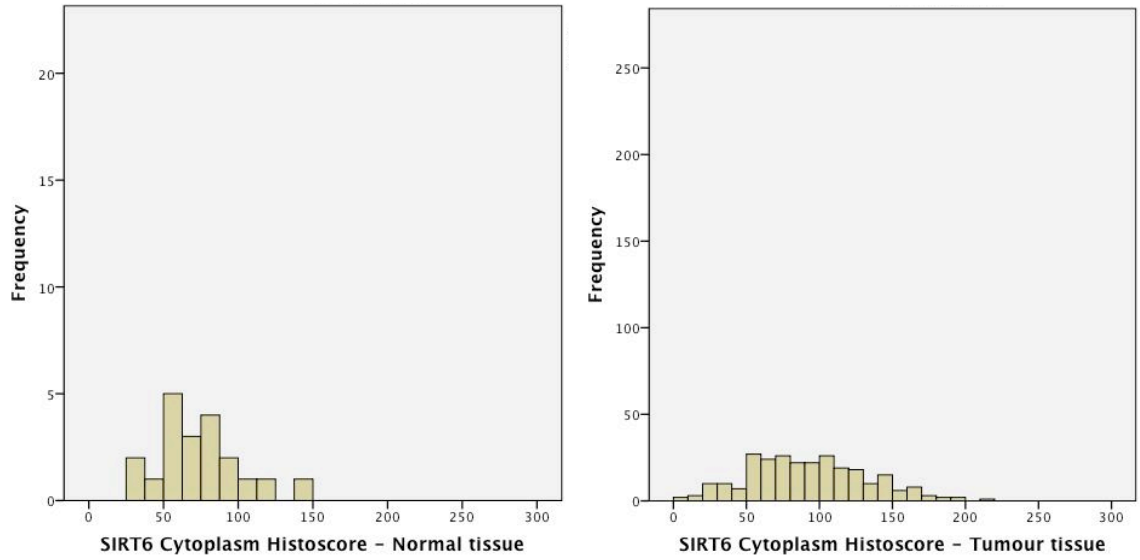

Figure 4.38 Two histograms show the frequency distribution of SIRT6 Histoscores in cytoplasm, in normal and tumour tissue samples.

Staining intensity was generally higher in tumour than normal tissue; this was not significantly different on related samples Wilcoxon Signed Rank test (p=0.211). Comparison of the distribution of Histoscores in the full cohort of tumour samples did show a significant difference (Mann-Whitney U test, p=0.0278). Thus levels of SIRT6 protein in the cytoplasm were higher in colorectal tumour cells than in normal colorectal cells.

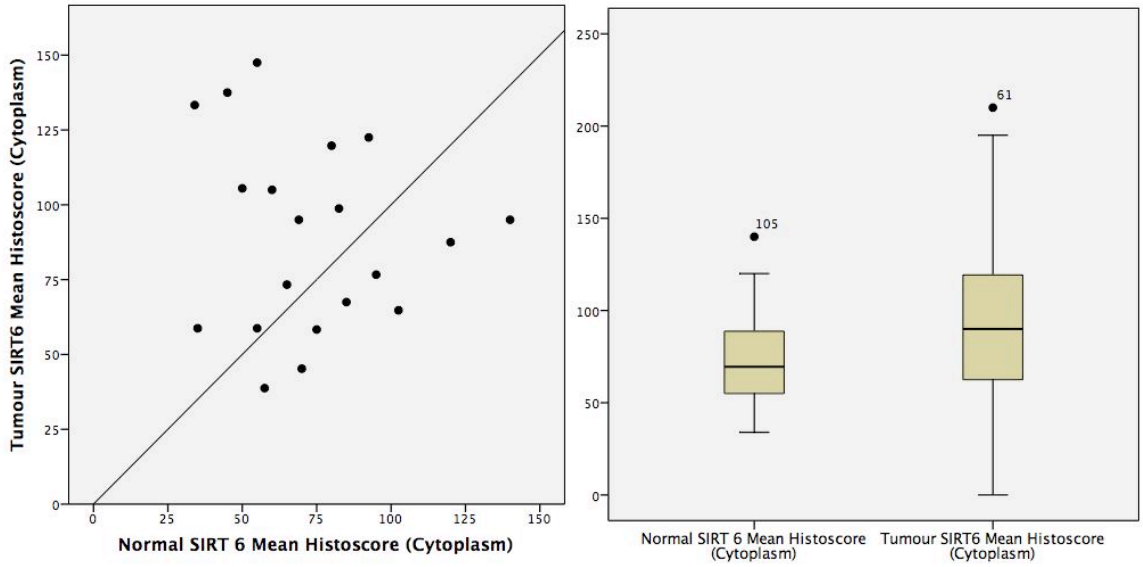

Figure 4.39 Scatterplot shows SIRT6 cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed lower Histoscore in normal than in tumour tissue. The boxplots of SIRT6 cytoplasmic Histoscores from the full cohort of colorectal tumour and normal tissue samples showed higher Histoscores in tumour tissue (Mann-Whitney U test, p=0.0278).

4.7 SIRT7 Immunohistochemistry

Representative pictures of sections stained for SIRT7 are shown.

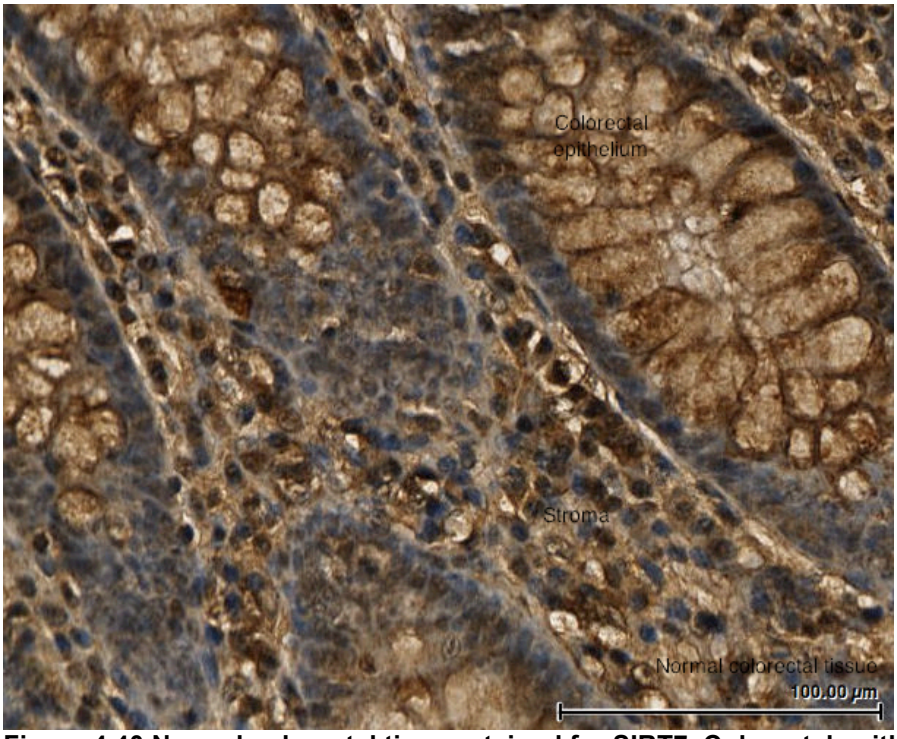

Figure 4.40 Normal colorectal tissue stained for SIRT7. Colorectal epithelium and stroma are labelled.

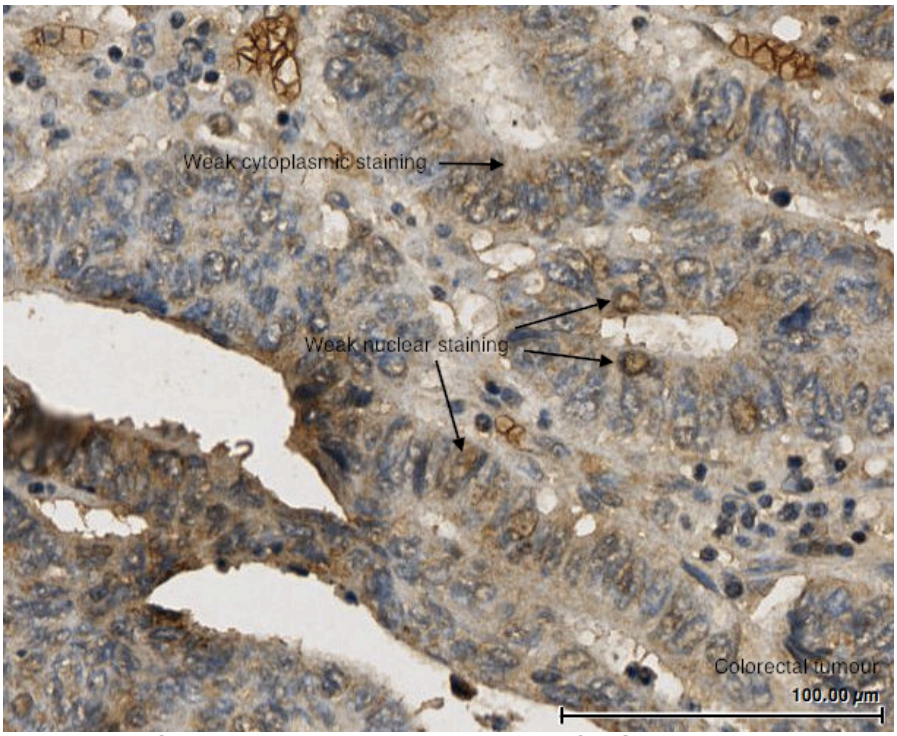

Figure 4.41 Colorectal tumour tissue stained for SIRT7. In this example, cytoplasmic staining is weak (arrows) where present. Nuclear staining is also weak as indicated by arrows.

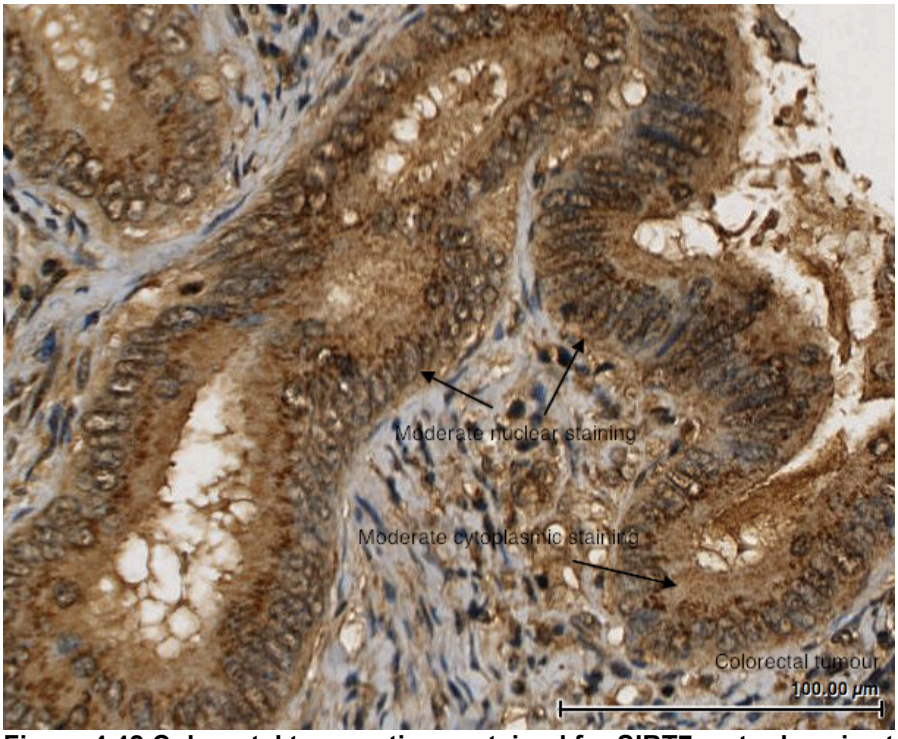

Figure 4.42 Colorectal tumour tissue stained for SIRT7; cytoplasmic staining is stronger in this section and nuclear staining is also more prominent (as indicated by arrows).

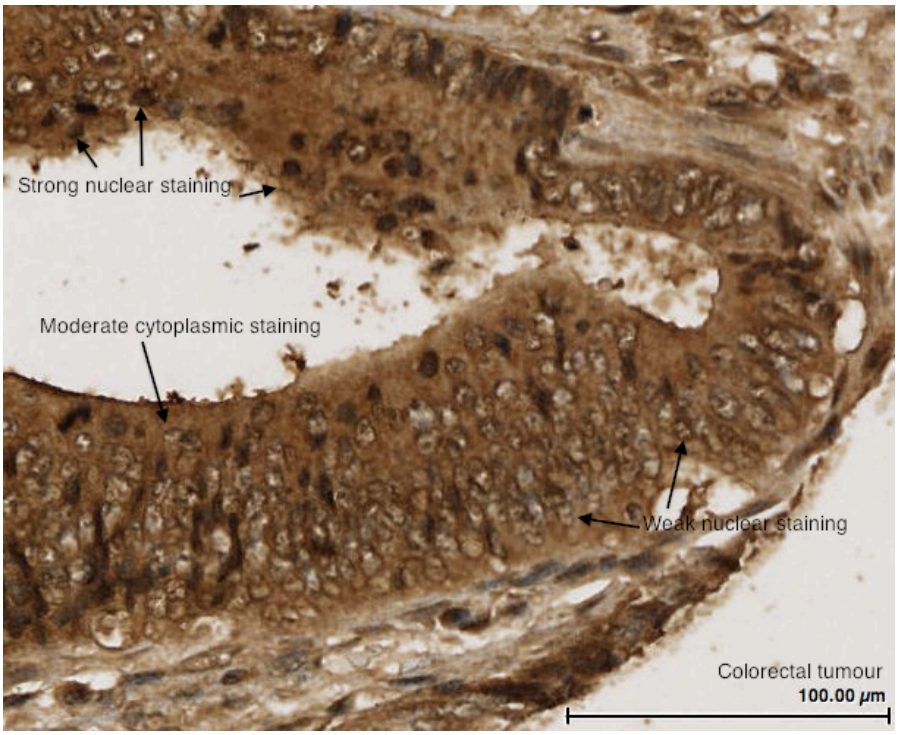

Figure 4.43 Colorectal tissue stained for SIRT7. Cytoplasmic staining is once again moderate and shows patchy variation. Nuclear staining varies between weak and very strong.

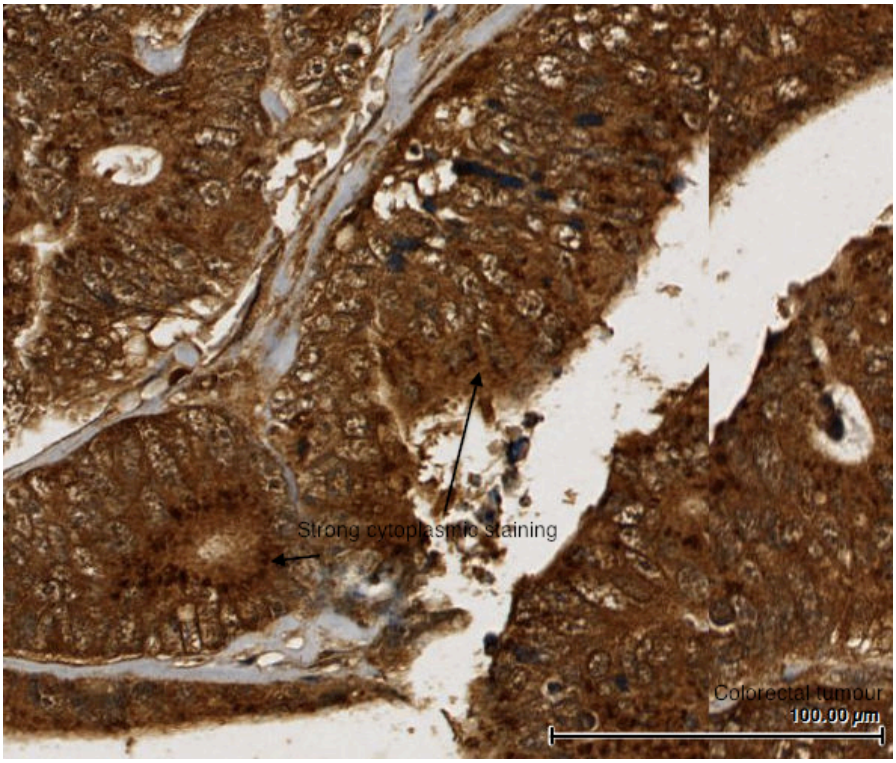

Figure 4.44 Colorectal tumour tissue stained for SIRT7. Cytoplasmic staining is very strong and shows a granular pattern in some areas. Nuclear staining by contrast is quite weak.

Basic descriptive statistics for SIRT7 staining are given below.

SIRT7 Nuclear Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**20.00**	**28.13**	**5.00**	**142.50**	**10.00**	**37.50**
Tumour tissue	**32.50**	**37.50**	**0.00**	**146.67**	**15.00**	**52.50**
SIRT7 Cytoplasmic Histoscore	Median	Interquartile range	Minimum	Maximum	Lower quadrant	Upper quadrant
Normal tissue	**125.00**	**36.88**	**100.00**	**175.00**	**115.00**	**150.00**
Tumour tissue	**112.50**	**31.25**	**41.25**	**265.00**	**98.75**	**130.00**

Table 4.6 Descriptive statistics for SIRT7 Histoscores in nuclei and cytoplasm, in normal colorectal epithelium and tumour tissue.

4.7.1 SIRT7 protein expression levels in the nucleus in normal and tumour tissue.

Distribution of SIRT7 Histoscores in nuclei is represented graphically below for both normal and tumour tissue.

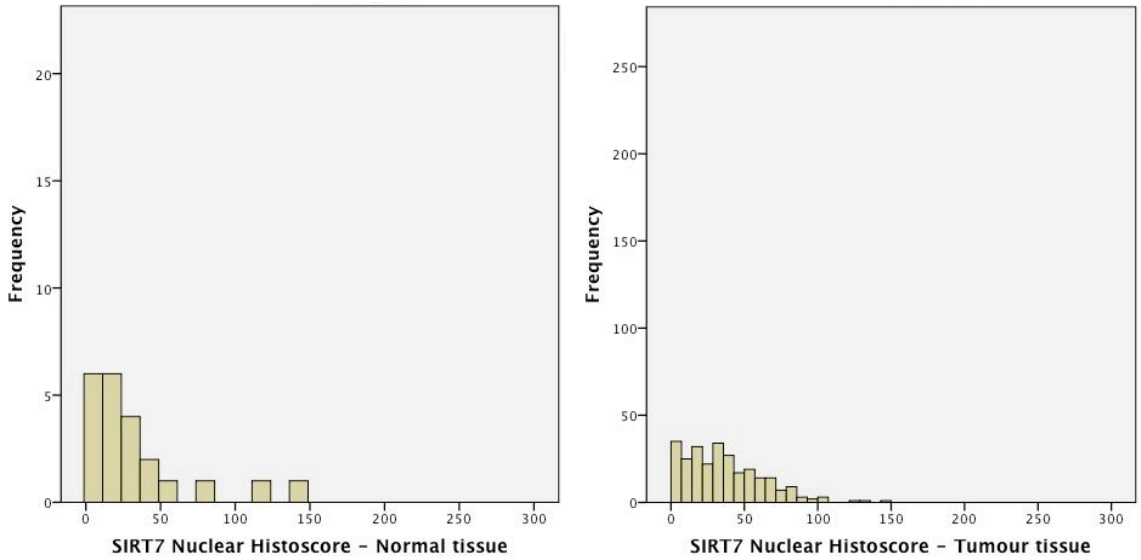

Figure 4.45 Two histograms showing the frequency distribution of SIRT7 Histoscores in nuclei, in normal and tumour tissue samples.

Related samples Wilcoxon Signed Rank test (p=0.6377) and Mann-Whitney U test (p=0.151) comparing the distribution of Histoscores in matched and unmatched samples showed there was no significant difference in staining between normal colorectal and colorectal tumour tissue.

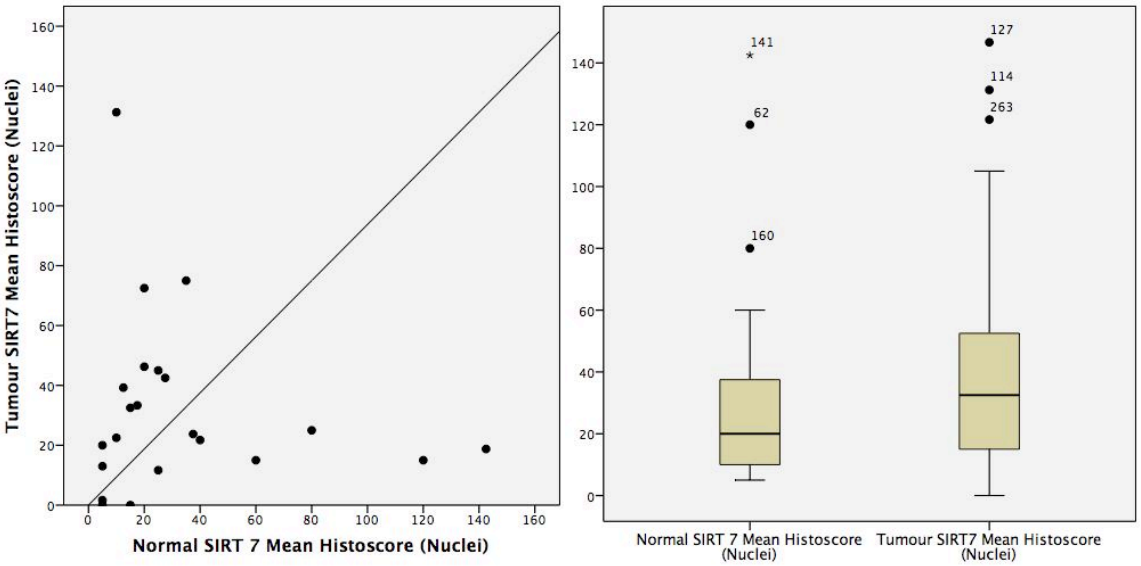

Figure 4.46 Scatterplot shows SIRT7 nuclear Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Histoscores in matched tumour and normal tissue samples were similar Wilcoxon Signed Rank test (p=0.6377). The boxplots of SIRT7 nuclear Histoscores from the full cohort of colorectal tumour and normal tissue samples showed similar Histoscores in both tissues (Mann-Whitney U test, p=0.151).

4.7.2 SIRT7 protein expression levels in the cytoplasm in normal and tumour tissue.

Distribution of SIRT7 Histoscores in cytoplasm is represented graphically below for both normal and tumour tissue.

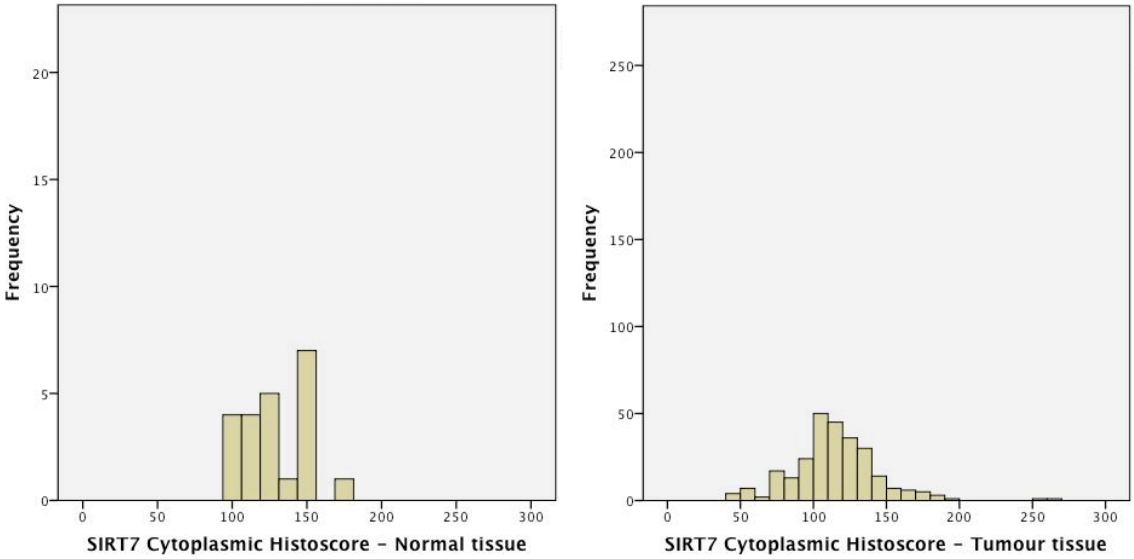

Figure 4.47 Two histograms show the frequency distribution of SIRT7 Histoscores in cytoplasm, in normal and tumour tissue samples.

Staining intensity was generally lower in tumour than normal tissue; this was not significantly different on related samples Wilcoxon Signed Rank test (p=0.079). Comparison of the distribution of Histoscores in the full cohort of tumour samples did show a significant difference (Mann-Whitney U test, p=0.009). Thus levels of SIRT7 protein in the cytoplasm were lower in colorectal tumour cells than in normal colorectal cells.

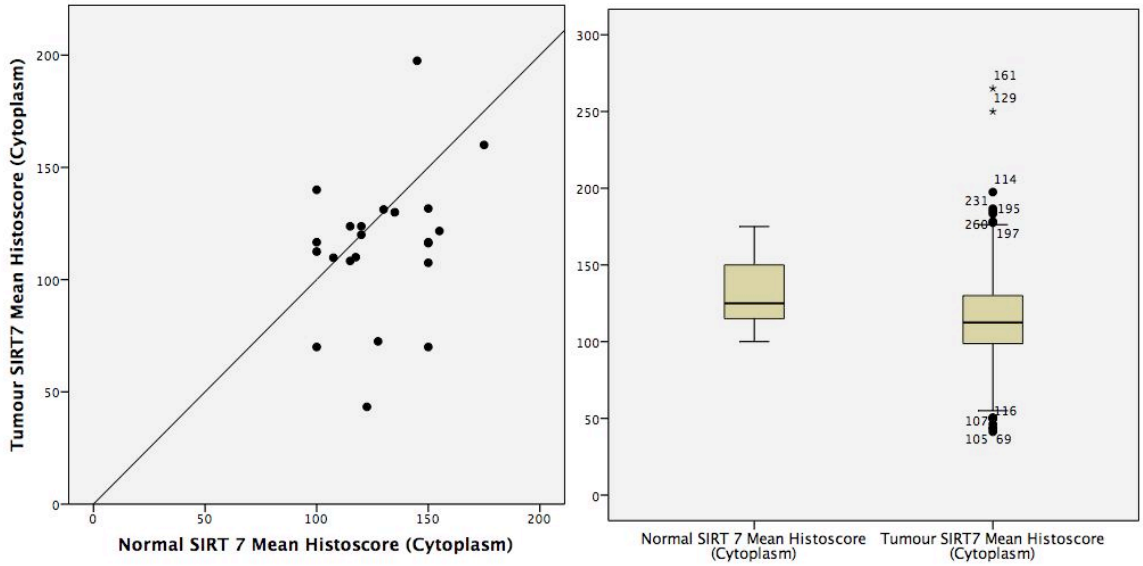

Figure 4.48 Scatterplot shows SIRT7 cytoplasmic Histoscores for matched samples from colorectal tumour tissue and adjacent normal colorectal tissue. The solid line is the line of equivalence. Most points showed higher Histoscore in normal than in tumour tissue. The boxplots of SIRT7 cytoplasmic Histoscores from the full cohort of colorectal tumour and normal tissue samples showed lower Histoscores in tumour tissue (Mann-Whitney U test, p=0.009).

These results will be discussed in the context of clinicopathological associations with sirtuin expression, outlined in Chapter 5. Each sirtuin is considered separately, with analysis of sirtuin interrelationships in Chapter 6.

4.8 Effect of sirtuin expression in colorectal tumour tissue on cancer-specific survival.

Survival data was collated by Mr James Park at December 2011, a period at least 5 years from operation for all cases in the database. Cause of death was identified as colorectal cancer-related or due to other cause. Kaplan-Meier survival curves were produced for each combination of sirtuin (SIRT2-7) and location (nuclear or cytoplasmic), with cancer-related death as the outcome of interest. Histoscores were divided into quartiles and equality of survival distribution for each quartile was compared.

4.8.1 Effect of SIRT2 expression in colorectal tumour tissue on survival.

Kaplan-Meier analysis of survival was performed with SIRT2 nuclear Histoscore divided into quartiles. There was a significant difference between quartiles on log rank (Mantel-Cox) test ($p=0.0202$): from the Kaplan-Meier curves, lowest and highest quartiles of SIRT2 nuclear staining were associated with similarly worse outcomes than middle quartiles.

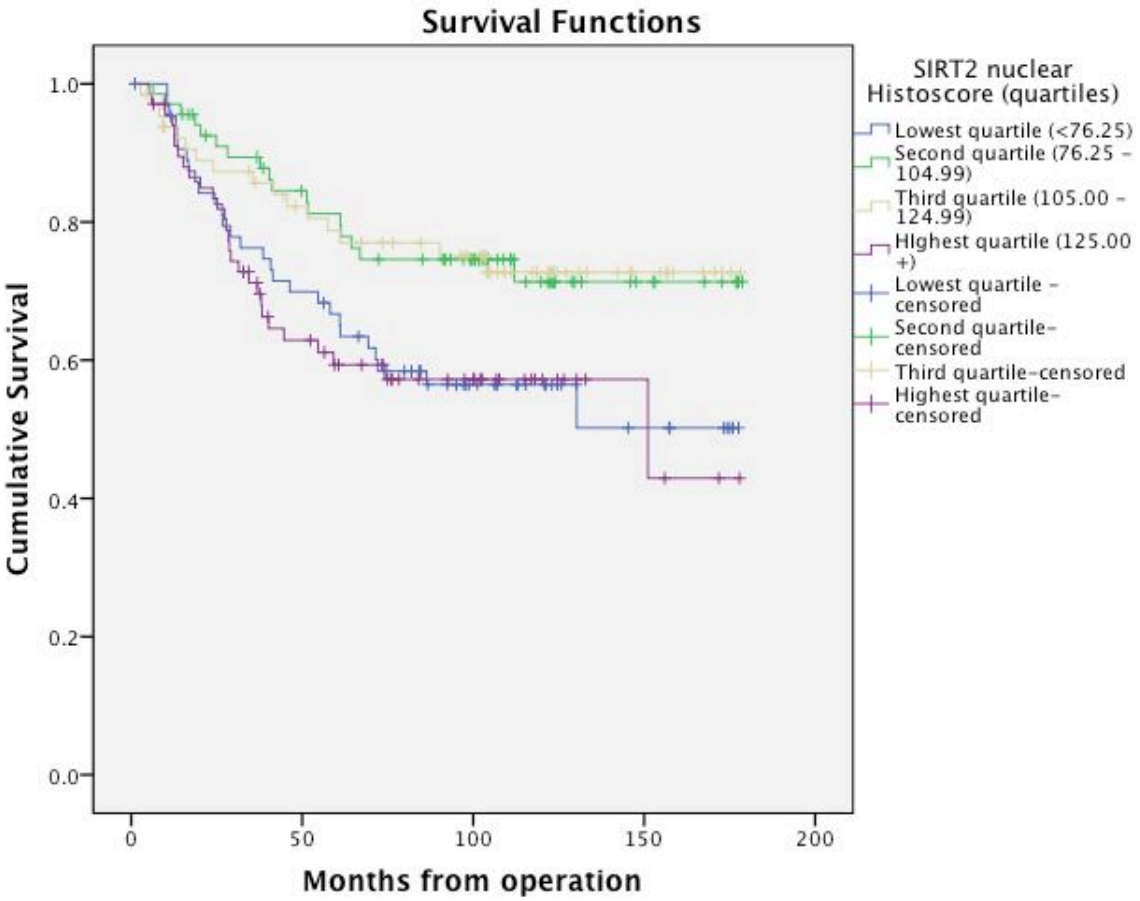

Figure 4.49 Kaplan-Meier survival curves for SIRT2 nuclear Histoscores split in quartiles. There was a significant difference between quartiles on log rank (Mantel-Cox) test (p=0.0202): from the Kaplan-Meier curves, lowest and highest quartile SIRT2 nuclear staining was associated with worse outcome.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	9.814	3	0.0202
Breslow (Generalized Wilcoxon)	9.137	3	0.0275
Tarone-Ware	9.407	3	0.0243

Table 4.7 Tests of equality of survival distributions for the different levels of SIRT2 nuclear Histoscore (split in quartiles).

Lowest and highest quartile SIRT2 nuclear Histoscores were combined and survival analysis was performed on the 'extreme quartiles' versus the middle two quartiles. The difference between the two groups in terms of survival, on log rank (Mantel-Cox) test, was more statistically significant again (p=0.0018).

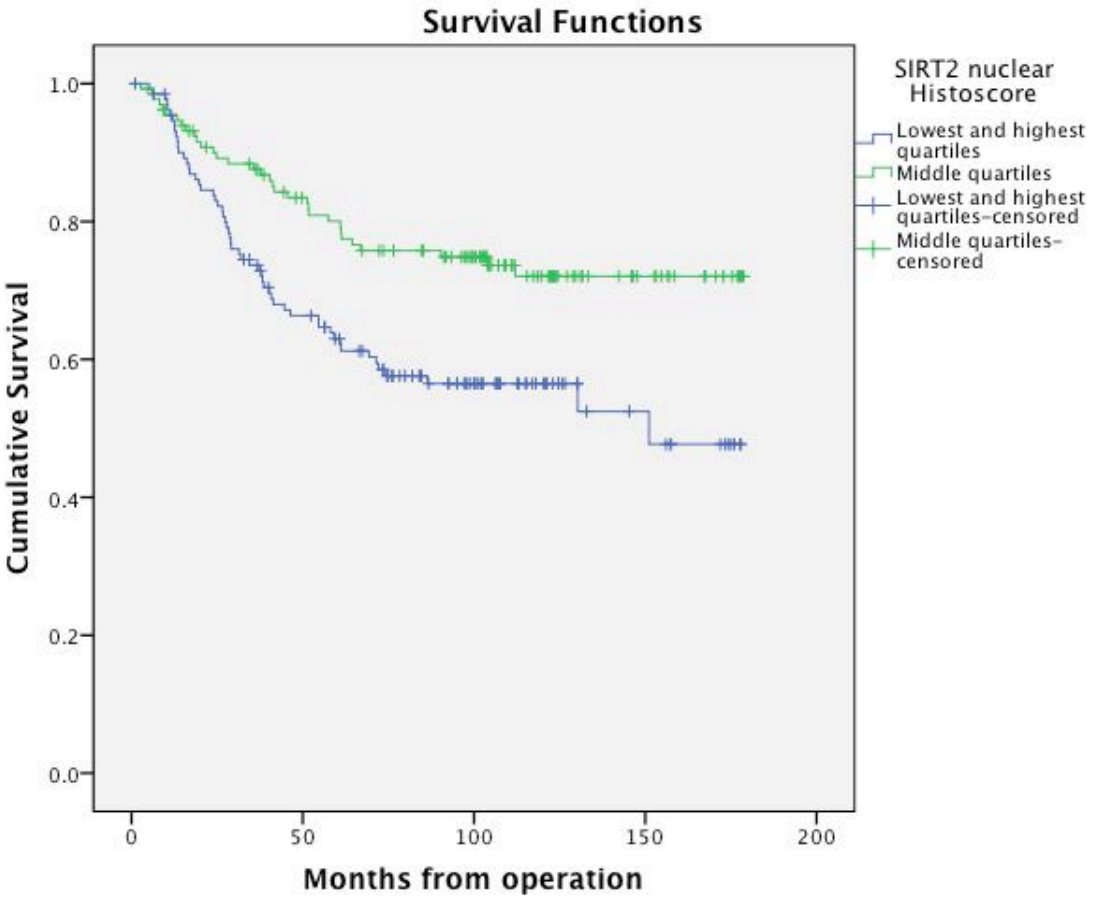

Figure 4.50 Kaplan-Meier survival curves for SIRT2 nuclear Histoscores, split lowest and highest quartiles versus middle quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	9.702	1	0.0018
Breslow (Generalized Wilcoxon)	8.928	1	0.0028
Tarone-Ware	9.262	1	0.0023

Table 4.8 Tests of equality of survival distributions for the different levels of SIRT2 nuclear Histoscore (split lowest and highest quartiles versus middle quartiles).

Kaplan-Meier analysis of survival was performed with SIRT2 cytoplasmic Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

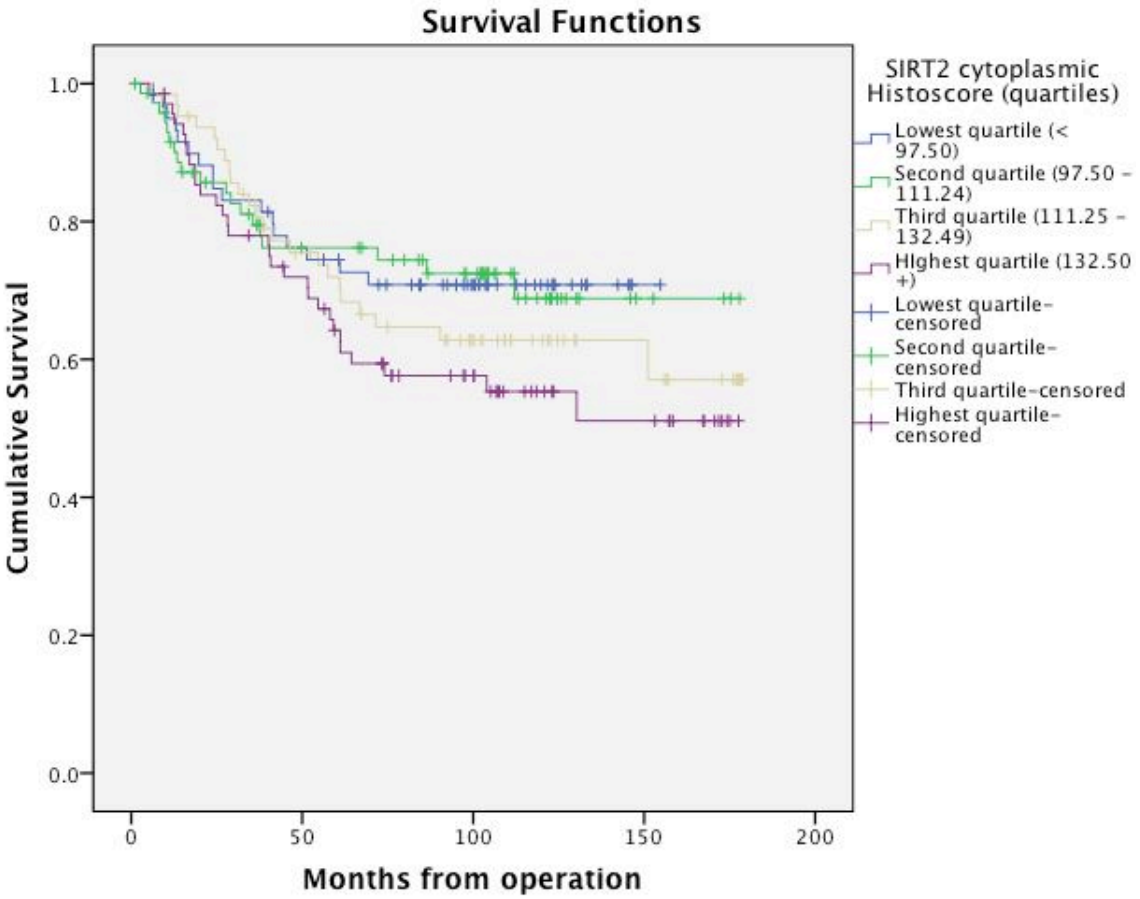

Figure 4.51 Kaplan-Meier survival curves for SIRT2 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.469	3	0.3248
Breslow (Generalized Wilcoxon)	2.310	3	0.5105
Tarone-Ware	2.843	3	0.4164

Table 4.9 Tests of equality of survival distributions for the different levels of SIRT2 cytoplasmic Histoscores (split in quartiles).

4.8.2 Effect of SIRT3 expression in colorectal tumour tissue on survival.

Kaplan-Meier analysis of survival was performed with SIRT3 nuclear Histoscore divided into quartiles. SIRT3 nuclear staining was present in less than half the cases and weak, where present. There was therefore no way to divide cases into lowest quartile and second quartile and these were amalgamated into one group. There was a difference between groups on log rank (Mantel-Cox) test, although this did not reach significance.

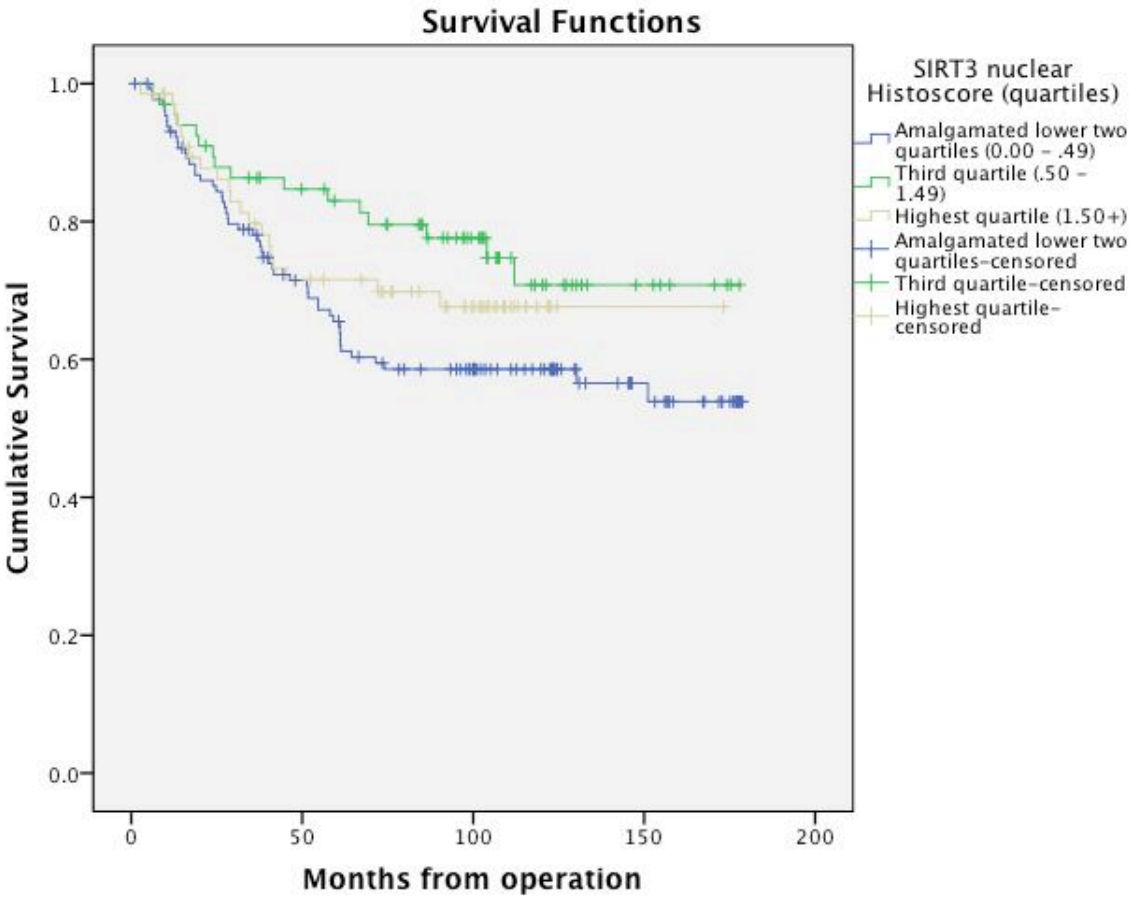

Figure 4.52 Kaplan-Meier survival curves for SIRT3 nuclear Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	5.235	2	0.0730
Breslow (Generalized Wilcoxon)	5.374	2	0.0681
Tarone-Ware	5.396	2	0.0673

Table 4.10 Tests of equality of survival distributions for the different levels of SIRT3 nuclear Histoscores (split in quartiles).

Kaplan-Meier survival curves and tests to compare the equality of survival distributions are included above, but given the characteristics of the data, this analysis (applied to the other sirtuins) may not be most suitable for SIRT3 nuclear Histoscore. Therefore, similar analysis was performed with SIRT3 nuclear Histoscore divided about the median. The difference in distribution of survival between the two groups was statistically significant on log rank (Mantel-Cox) test (p=0.0343).

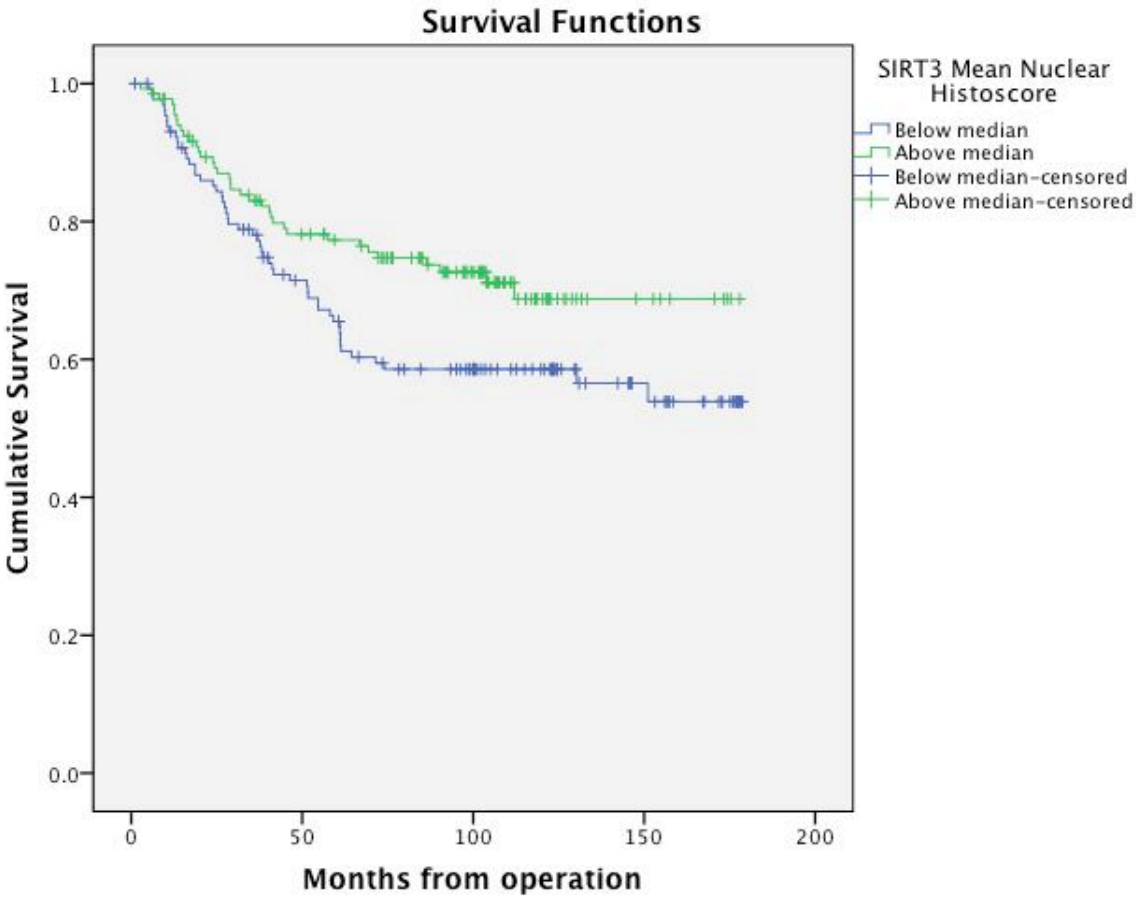

Figure 4.53 Kaplan-Meier survival curves for SIRT3 nuclear Histoscores split about the median.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.480	1	0.0343
Breslow (Generalized Wilcoxon)	4.343	1	0.0372
Tarone-Ware	4.466	1	0.0346

Table 4.11 Tests of equality of survival distributions for the different levels of SIRT3 nuclear Histoscores (split above and below the median).

Kaplan-Meier analysis of survival was performed with SIRT3 cytoplasmic Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

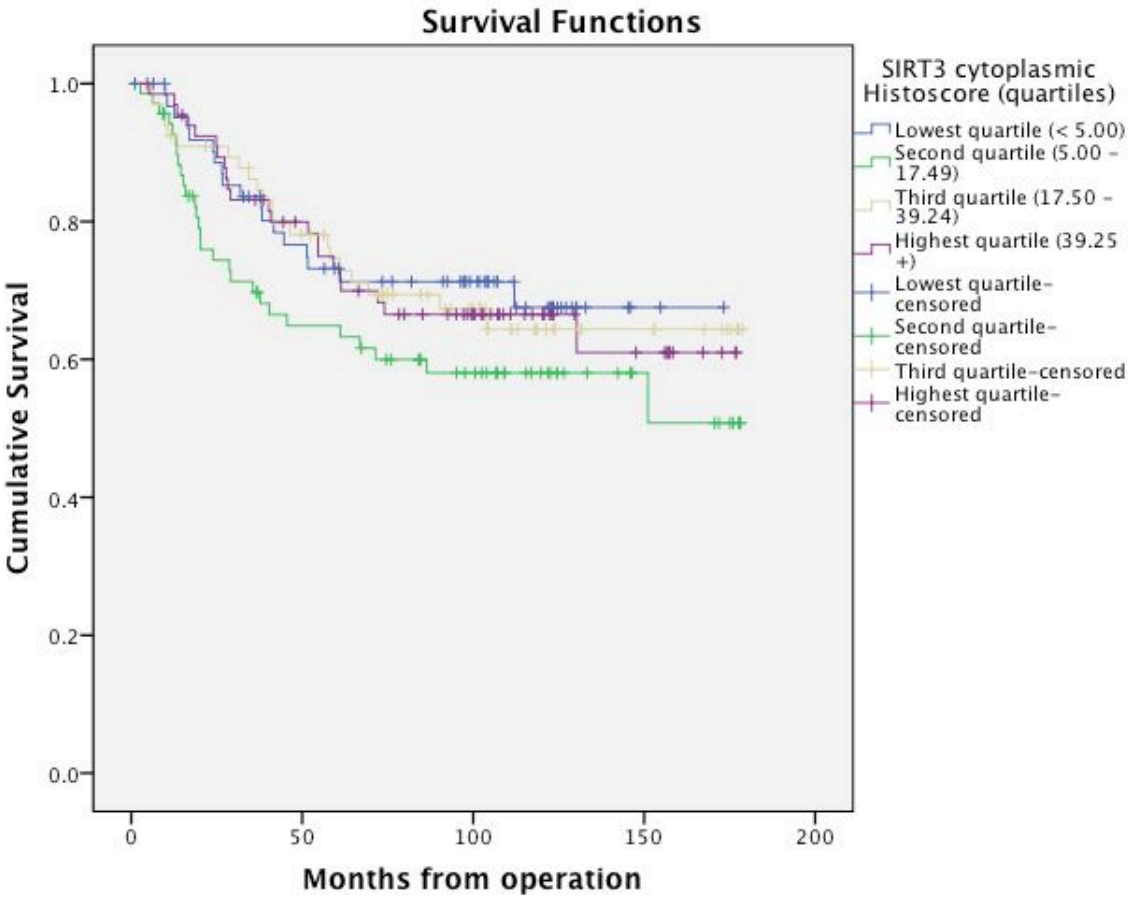

Figure 4.54 Kaplan-Meier survival curves for SIRT3 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	3.134	3	0.3714
Breslow (Generalized Wilcoxon)	4.092	3	0.2517
Tarone-Ware	3.599	3	0.3081

Table 4.12 Tests of equality of survival distributions for the different levels of SIRT3 cytoplasmic Histoscores (split in quartiles).

4.8.3 Effect of SIRT4 expression in colorectal tumour tissue on survival.

SIRT4 nuclear staining was weak and infrequent, and there was no way to differentiate cases into the lower three quartiles. Kaplan-Meier analysis of survival was therefore performed with SIRT4 nuclear Histoscore divided into lowest three quartiles and highest quartile. There was no significant difference in equality of survival distribution between the two groups.

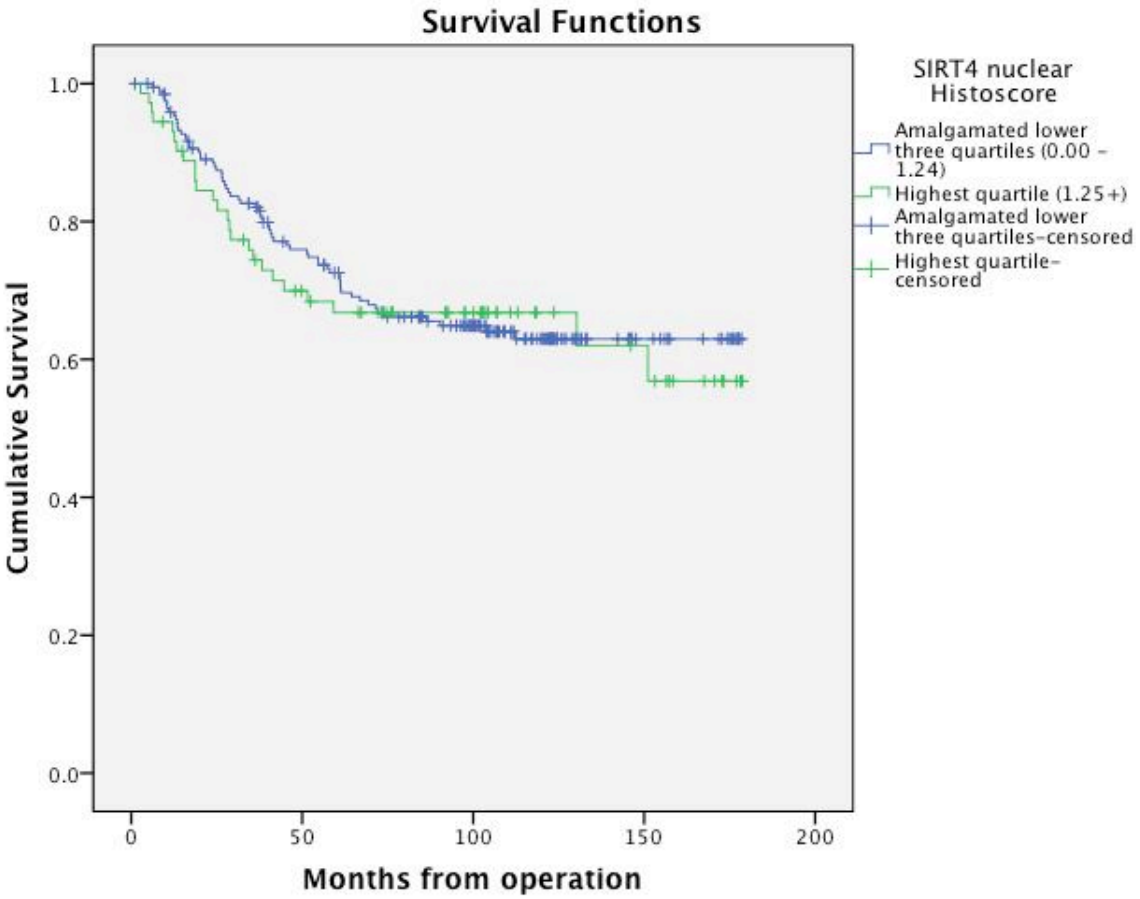

Figure 4.55 Kaplan-Meier survival curves for SIRT4 nuclear Histoscores divided into lowest three quartiles and highest quartile.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.102	1	0.7490
Breslow (Generalized Wilcoxon)	.229	1	0.6321
Tarone-Ware	.131	1	0.7174

Table 4.13 Tests of equality of survival distributions for the different levels of SIRT4 nuclear Histoscores (divided into lowest three quartiles and highest quartile).

Kaplan-Meier analysis of survival was performed with SIRT4 cytoplasmic Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

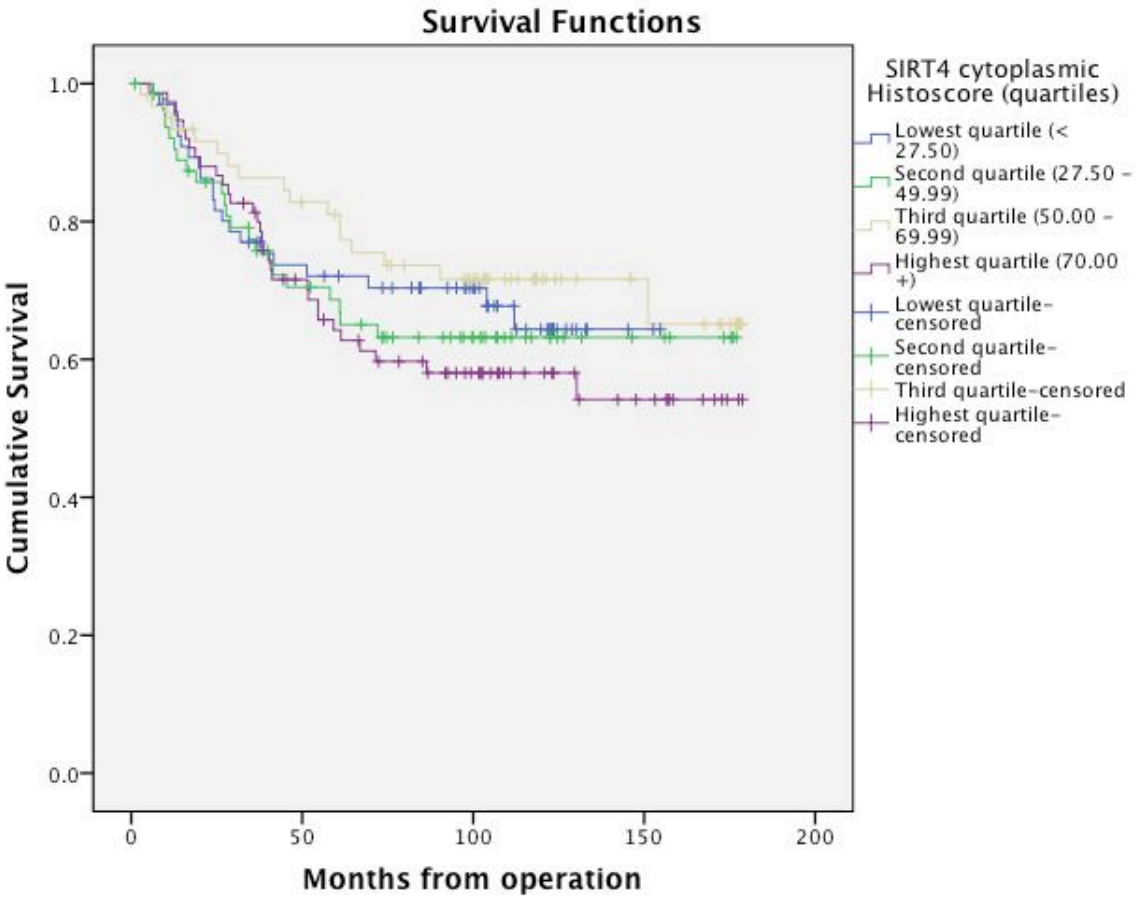

Figure 4.56 Kaplan-Meier survival curves for SIRT4 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	2.462	3	0.4822
Breslow (Generalized Wilcoxon)	2.485	3	0.4780
Tarone-Ware	2.554	3	0.4656

Table 4.14 Tests of equality of survival distributions for the different levels of SIRT4 cytoplasmic Histoscores (split in quartiles).

4.8.4 Effect of SIRT5 expression in colorectal tumour tissue on survival.

SIRT5 nuclear staining was present in only 32 cases, and was thus so infrequent that it was not possible to divide cases into quartiles for comparison of survival.

Kaplan-Meier analysis of survival was performed with SIRT5 cytoplasmic Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

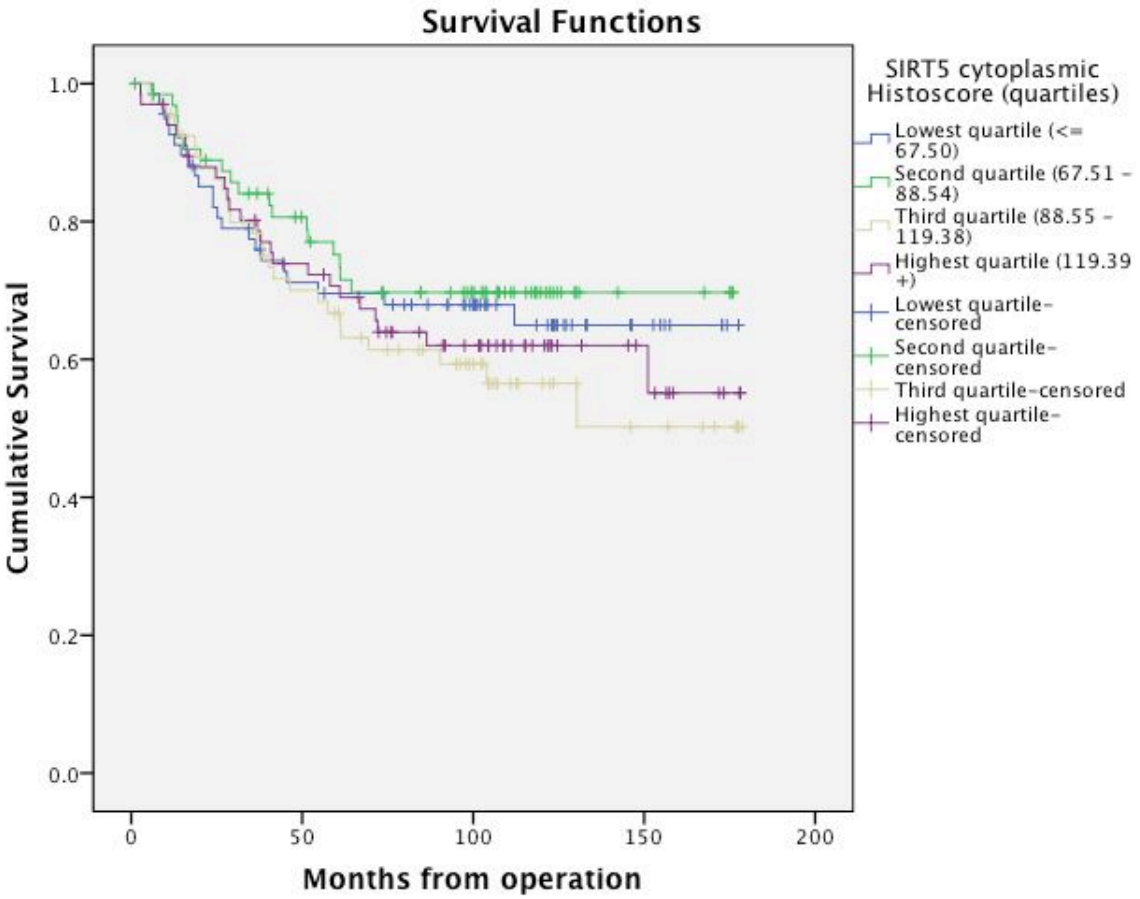

Figure 4.57 Kaplan-Meier survival curves for SIRT5 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	2.129	3	0.5460
Breslow (Generalized Wilcoxon)	1.398	3	0.7059
Tarone-Ware	1.665	3	0.6448

Table 4.15 Tests of equality of survival distributions for the different levels of SIRT5 cytoplasmic Histoscores (split in quartiles).

Figure 4.58 Tests of equality of survival distributions for the different levels of SIRT5 cytoplasmic Histoscores (split in quartiles).

4.8.5 Effect of SIRT6 expression in colorectal tumour tissue on survival.

Kaplan-Meier analysis of survival was performed with SIRT6 nuclear Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

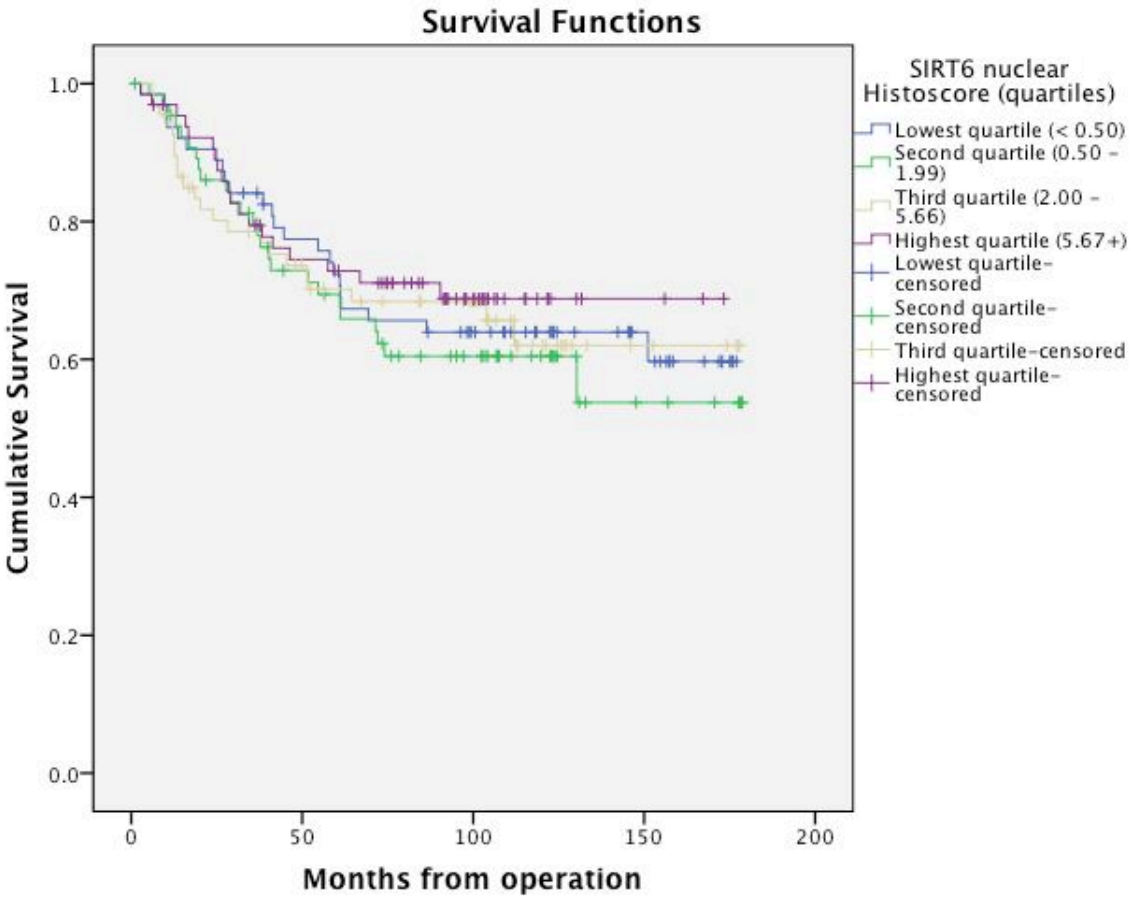

Figure 4.59 Kaplan-Meier survival curves for SIRT6 nuclear Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.946	3	0.8144
Breslow (Generalized Wilcoxon)	.709	3	0.8710
Tarone-Ware	.788	3	0.8523

Table 4.16 Tests of equality of survival distributions for the different levels of SIRT6 nuclear Histoscores (split in quartiles).

Kaplan-Meier analysis of survival was performed with SIRT6 cytoplasmic Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

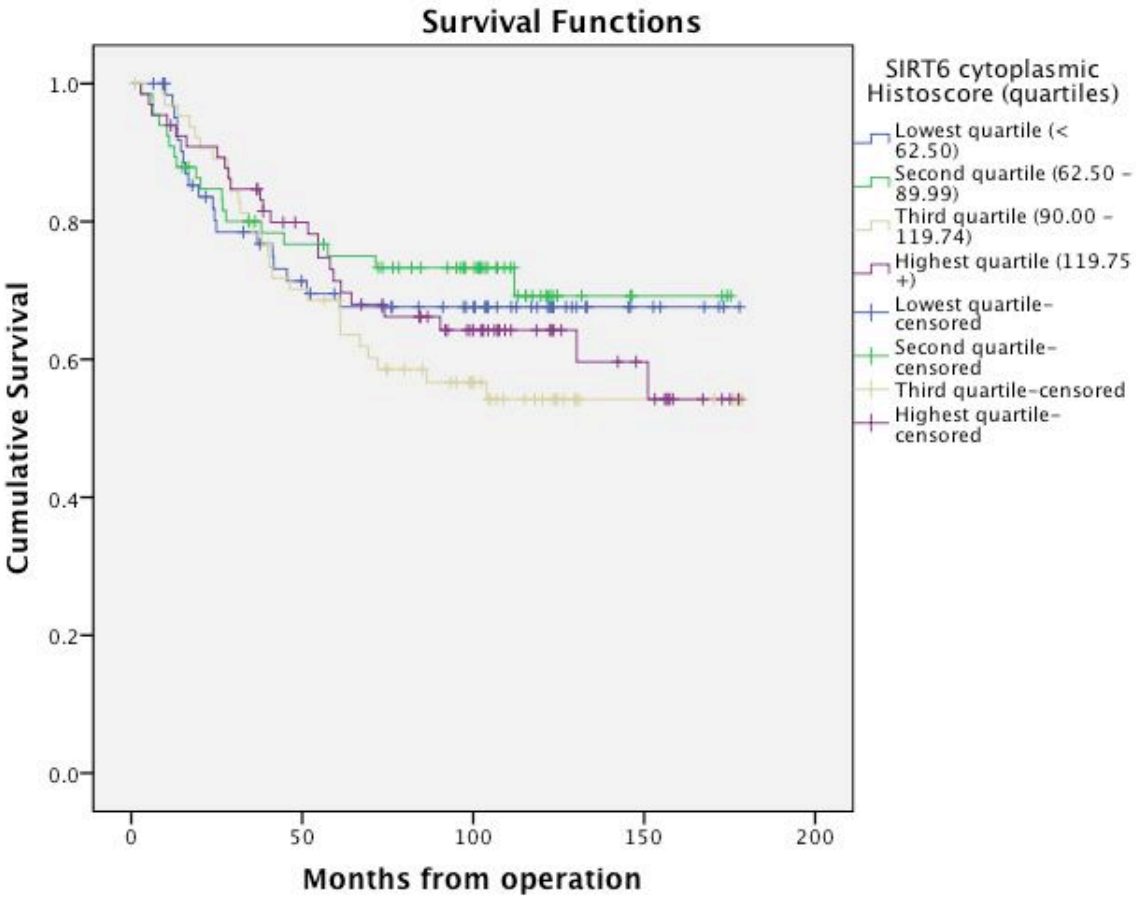

Figure 4.60 Kaplan-Meier survival curves for SIRT6 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	2.260	3	0.5202
Breslow (Generalized Wilcoxon)	1.193	3	0.7547
Tarone-Ware	1.690	3	0.6391

Table 4.17 Tests of equality of survival distributions for the different levels of SIRT6 cytoplasmic Histoscores (split in quartiles).

4.8.6 Effect of SIRT7 expression in colorectal tumour tissue on survival.

Kaplan-Meier analysis of survival was performed with SIRT7 nuclear Histoscore divided into quartiles. There was no significant difference in equality of survival distribution between quartiles.

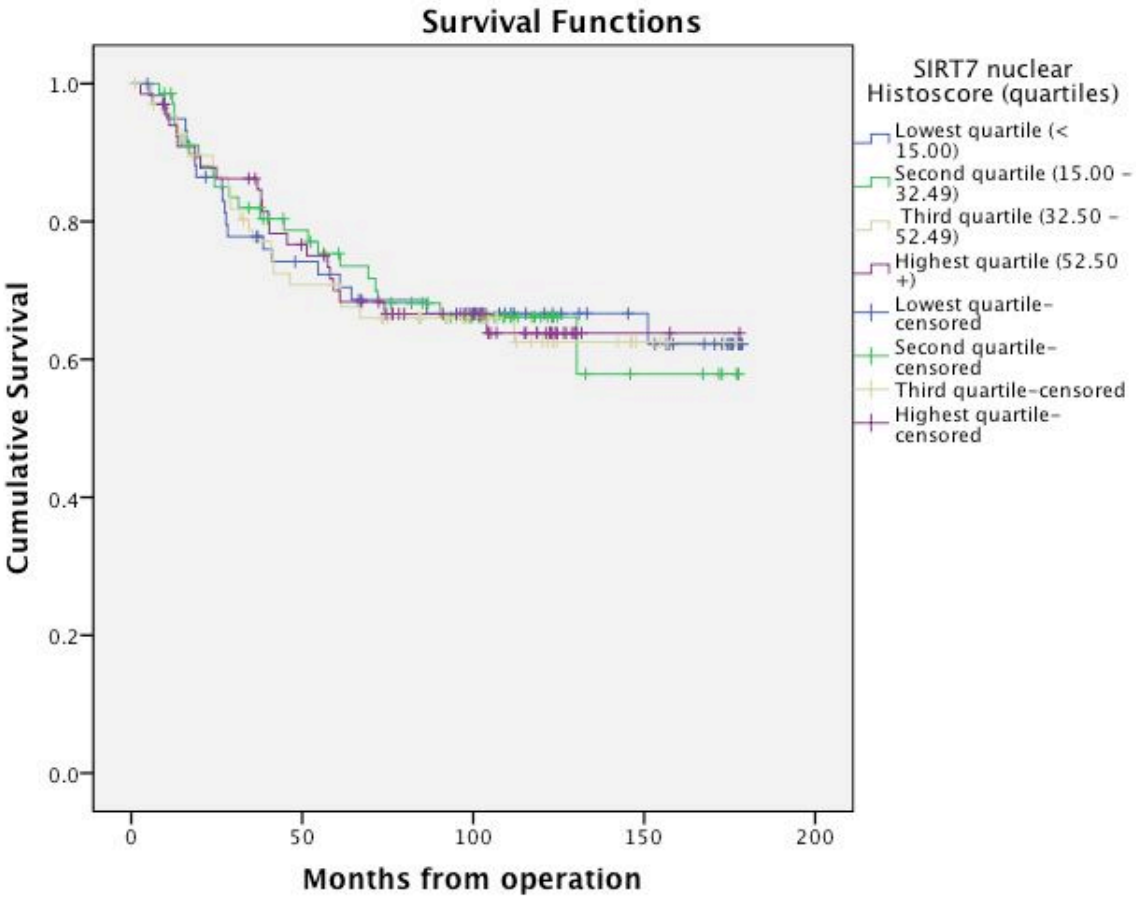

Figure 4.61 Kaplan-Meier survival curves for SIRT7 nuclear Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.031	3	0.9985
Breslow (Generalized Wilcoxon)	.110	3	0.9906
Tarone-Ware	.070	3	0.9951

Table 4.18 Tests of equality of survival distributions for the different levels of SIRT7 nuclear Histoscores (split in quartiles).

Kaplan-Meier analysis of survival was performed with SIRT7 cytoplasmic Histoscore divided into quartiles. There was a significant difference between quartiles on log rank (Mantel-Cox) test (p=0.0088). There was no incremental relationship between SIRT7 cytoplasmic Histoscore by quartile and survival. There was no relationship between extremes of expression and survival, unlike the relationship with SIRT2 nuclear Histoscore.

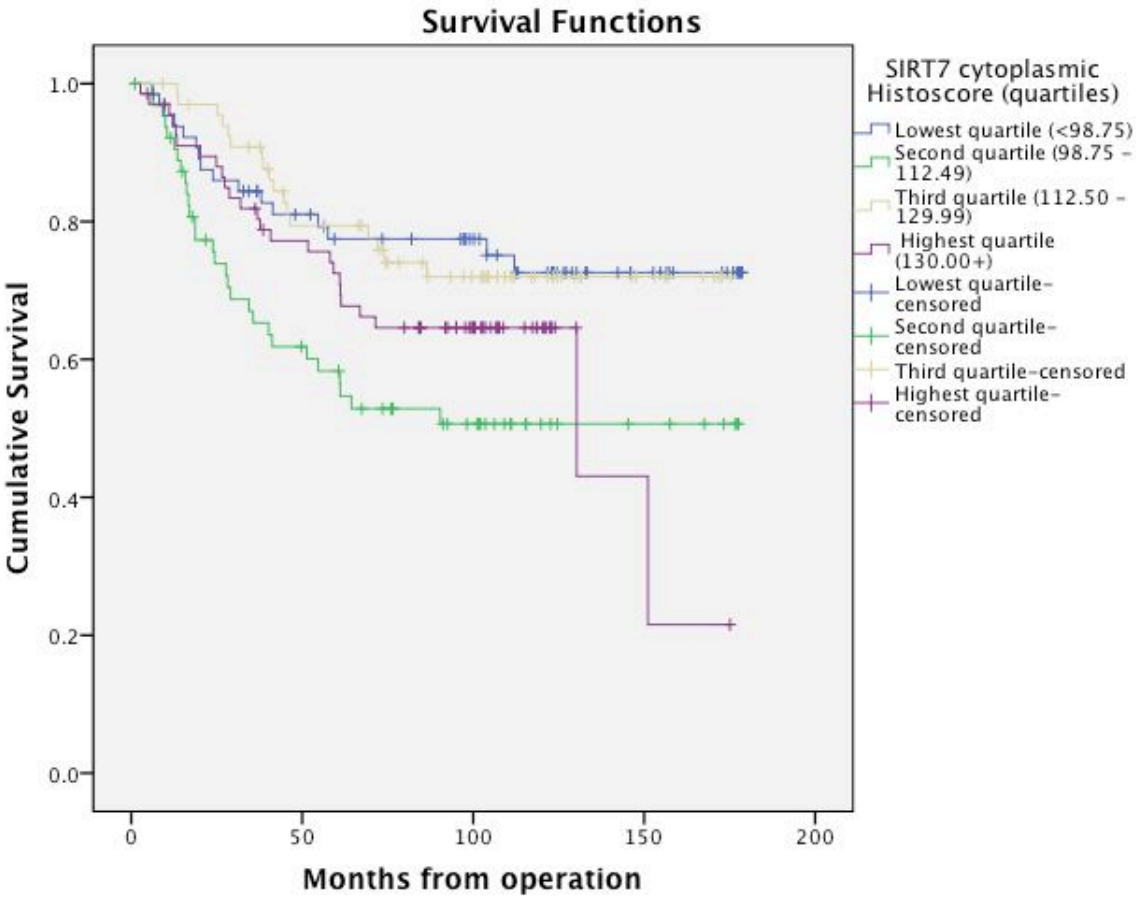

Figure 4.62 Kaplan-Meier survival curves for SIRT7 cytoplasmic Histoscores split in quartiles.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	11.631	3	0.0088
Breslow (Generalized Wilcoxon)	12.768	3	0.0052
Tarone-Ware	12.307	3	0.0064

Table 4.19 Tests of equality of survival distributions for the different levels of SIRT7 cytoplasmic Histoscores (split in quartiles).

4.9 Regression analysis of effect of sirtuin expression on survival

Univariate survival analysis was performed using Cox's proportional hazards model, for Histoscores for each sirtuin and subcellular combination where Kaplan-Meier analysis of Histoscore quartiles showed a significant effect on equality of survival distribution. Univariate survival analysis was also performed for variables with accepted association with outcome from colorectal cancer. Data was available for smoking status for only 80% of cases and platelet count for only half the cases, so these variables were excluded from the analysis. Included variables and the corresponding p-values are listed in the figure below.

Variables with significant effect according to univariate survival analysis were included in a multivariate survival analysis also using Cox's proportional hazards model. Composite variables were not included. Including composite variables has the effect of amplifying the effect their component variables have on the statistical analysis. Thus, of the variables identified as having a significant effect on survival in univariate analysis, the following were excluded: mGPS, Dukes' stage and peritoneal involvement. The first of these are composite measures whose components have an effect on colorectal cancer outcome in their own right. Peritoneal involvement is part of the defining criteria for T stage. T stage was included instead to allow for the other aspects of local invasion that it measures over and above peritoneal involvement.

A threshold p-value of less than or equal to 0.1 was used as the criterion for inclusion in the multivariate model. A stepwise backward procedure was used to derive a model of variables with independent relationship with survival. Variables were removed if the corresponding p-value was greater than 0.05.

Nodal status (reflected in N stage), higher CRP, and presence of venous invasion and tumour perforation were independently associated with lower survival in this multivariate model. These are accepted risk factors which support the assumption that this is a cohort of characteristic colorectal cancer patients. Highest and lowest quartiles of SIRT2 nuclear protein levels are a novel risk factor associated with lower survival.

Peritumoural inflammation and higher SIRT3 nuclear protein levels show a trend towards an effect on survival.

VARIABLE	p-value
Age	0.209
BMI category	0.459
WCC	0.167
CRP	0.0052
Albumin	0.0042
mGPS	0.000024
Haemoglobin	0.585
Presentation (Elective or emergency)	0.058
SIRT2 Nuclear Histoscore categorised by extreme and middle quartiles	0.0022
SIRT2 Nuclear Histoscore categorised by quartiles	0.793
SIRT3 Nuclear Histoscore categorised about median	0.036
SIRT3 Nuclear Histoscore categorised absent or present	0.078
SIRT7 Cytoplasmic Histoscore categorised by quartiles	0.633
Dukes'	0.00004
T stage	0.0047
N stage	0.000018
M stage	0.275
Differentiation category	0.223
Margin Invasion	0.00079
Tumour Perforation	0.0083
Peritoneal Involvement	0.00036
Venous Invasion	0.000031
Petersen Index	0.0000000051
Peritumoural inflammation	0.014

Figure 4.63 Univariate survival analysis was performed for the variables listed above, and the corresponding p-values are included.

	p=value	Hazard Ratio	95.0% CI for Hazard Ratio	
			Lower	Upper
N stage	0.002	1.627	1.203	2.2
Venous Invasion	0.012	1.839	1.145	2.954
Tumour perforation	0.000325	5.162	2.109	12.634
CRP	0.002	2.01	1.281	3.156
Peritumoural inflammation	0.069	1.91	0.951	3.836
SIRT2 Nuclear Histoscore categorised by extreme quartiles	0.02	1.724	1.088	2.732
SIRT3 Nuclear Histoscore categorised about median	0.054	1.564	0.993	2.464

Figure 4.64 Variables with independent relationship with survival from colorectal cancer, with corresponding p-values and hazard ratios.

4.10 Discussion

Cellular events determine the development of a macroscopically evident tumour, which in turn contains a huge variety of cellular phenotypes. The measurements made in these investigations average over many cellular histories and current phenotypes, but they have revealed widespread alteration in protein expression on development of malignancy in colorectal tissue, and in the case of SIRT2, a robust effect on survival.

4.10.1 SIRT2 protein levels in normal and colorectal tumour tissue

There are 3 isoforms of SIRT2 shown to produce physiologically active proteins in humans. Not all of these have been demonstrated in all tissues, and none have been specifically demonstrated in colorectal tissue. The antibody used in these experiments did not distinguish between isoforms of SIRT2 (LifespanBiosciences 2010). Given the subsequent effect on survival it would be useful to know more about the relative distribution of each one.

SIRT2 nuclear and cytoplasmic Histoscores are generally lower in colorectal cancer specimens. There are no previous reports of SIRT2 protein expression in colorectal cancer for comparison. A sample of 23 glioblastoma samples showed a similar relationship in terms of cytoplasmic staining, but an opposite correlation for nuclear staining which was higher than normal in the tumour tissue (Imaoka, Hiratsuka et al. 2012). Kim et al used a qualitative scale and found that SIRT2 staining was lower in all of 36 samples of breast tumour compared with adjacent normal tissue, but did not specify the subcellular localisation of the staining (Kim, Vassilopoulos et al. 2011). They also reported on 264 cases of HCC, of which 229 samples showed reduced staining for SIRT2 with respect to a group of unmatched normal samples, 125 showed no change, and 10 cases showed increased staining.

In a cohort of 374 cases of oesophageal adenocarcinoma, SIRT2 was identified as one of three dysregulated biomarkers which conferred poorer prognosis(Ong, Shapiro et al. 2013). SIRT2 staining was only measured in cytoplasm and the ‘dysregulated’ result was defined as low-level staining with respect to normal tissue (<10% cells positive).

SIRT2 mRNA levels were reduced in a range of human cancers (anaplastic oligodendroglioma, glioblastoma, clear cell renal carcinoma, and prostate carcinoma). SIRT2 mRNA levels were increased in 11 myeloid leukaemia cases(Dan, Klimenkova et al. 2012).

Given the small number of studies, the variability of tissue types and form of SIRT2 expression measured (mRNA or protein) and the dearth of precise subcellular localisation data contained in them, it is not possible to comment simply on whether these results are in keeping with other data. It is notable though that there is a tendency for reduced SIRT2 mRNA expression and protein levels across a wide range of cancers and the data presented here are broadly in keeping with this trend.

4.10.2 SIRT2 tumour Histoscore and association with survival

SIRT2 nuclear Histoscores in lowest and highest quartile are associated with worse survival in colorectal cancer in this cohort. While this pattern is common in many physiological measurements where homeostatic mechanisms are designed to keep parameters within a 'normal' range, it is slightly more difficult to interpret the significance of this observation against a background of generally reduced SIRT2 nuclear Histoscore with respect to normal tissue. The matched cohort on which this observation is based was very small.

The only other study identified linking SIRT2 nuclear protein levels with survival is referred to above (Kim, Vassilopoulos et al. 2011) and used glioblastoma specimens. These investigators found an inverse relationship: high SIRT2 nuclear protein levels correlated with worse survival, and were higher than in normal tissue. This simpler relationship, with increasing SIRT2 nuclear protein expression associated with cancer, and a more marked increase associated with worse survival is easier to conceptualise.

SIRT2 function as a mitotic checkpoint sensor is an obvious focus for further research to understand the observed effects on survival described here. Overexpression mutants show prolonged mitotic phase(Nahhas, Dryden et al. 2007). Overexpression also increases multinucleation, but so does the expression of a catalytically inactive form of SIRT2, reflecting the association with worse

survival in particularly high or low levels of SIRT2 expression reported here(North and Verdin 2007). SIRT2 deficiency in mice causes more mitotic abnormalities, aneuploidy and cell death(Kim, Vassilopoulos et al. 2011).

There are several SIRT2 isoforms in existence, each of which may have several and different actions in the nucleus. The shorter isoform is associated with reduced cell survival(Nahhas, Dryden et al. 2007). Information linking isoforms with actions and substrates is needed. Understanding the balance of SIRT2 isoforms in normal tissue, the variability among individuals and how the isoform levels change in the development of cancer would allow us to begin to fully understand the effect of SIRT2 on survival. However, the current results do highlight the significance of this protein as a useful focus for research and a possible therapeutic target in colorectal cancer and potentially in other malignancies as well.

4.10.3 SIRT3 protein levels in normal and colorectal tumour tissue

The antibody used in these experiments did not differentiate between the long and short isoforms of the SIRT3 protein. The datasheet notes that it was developed in response to a peptide corresponding to the human SIRT3 sequence(CellSignalling 2010), but it is not clear if this identifies a specific part of the protein. This is relevant as there is evidence to suggest that both long and short forms are found in the nucleus(Scher, Vaquero et al. 2007), and an antibody recognising only the short form may give a falsely low reading of SIRT3 nuclear protein expression. However this team did note very low endogenous protein levels for SIRT3, which is in keeping with the results found here.

This is the first reported account of SIRT3 protein expression in normal colorectal and colorectal tumour tissue. Of note, in comparison with other sirtuins, absolute Histoscore (and by inference, absolute protein levels) are very low. SIRT3 levels in the nucleus in both normal and tumour tissue are much lower than cytoplasmic expression. This is not indicative of level of importance of interactions with other proteins and may rather indicate specificity and precision of effect.

SIRT3 staining in both nuclear and cytoplasmic compartments are higher in tumour tissue, although this difference is only statistically significant in regard to cytoplasmic SIRT3. Previous work in this laboratory has noted reduced levels of SIRT3 mRNA in colorectal tumour samples with respect to matched normal tissue(Maxwell 2013). The alteration in protein expression in the opposite direction suggests significant differences in post-translational processing in normal and tumour tissue, which would bear further examination.

Depending on context, abundant SIRT3 could have a variety of different effects as indicated in a recent review on the place of SIRT3 in cancer cell function(Alhazzazi, Kamarajan et al. 2013). However, the presence of differential expression in tumour tissue highlights an anomaly worth investigation particularly in light of the worse survival associated with lower SIRT3 nuclear staining in tumour tissue.

4.10.4 SIRT3 tumour Histoscore and association with survival

SIRT3 cytoplasmic Histoscore was not associated with survival. Low SIRT3 nuclear Histoscore was associated with worse survival, although this effect did not reach significance on multivariable analysis. SIRT3 protein localisation to the nucleus is not entirely accepted in the field, but the weight of evidence for its presence and function there is growing(Scher, Vaquero et al. 2007; Iwahara, Bonasio et al. 2012). Through this observed effect on survival in a relatively large study, this data indicates the likelihood that SIRT3 has relevant functions in histone deacetylation and genetic silencing. It is of interest that Iwahara's team found that the long form of SIRT3 has a relatively short half-life in the nucleus, indicating that it can be controlled relatively tightly, possibly allowing for precise control of target gene expression.

A better understanding of the interactions which underlie the effect of SIRT3 on survival in univariate analysis, and the confounding factors which actually have the effect on survival, might open novel therapeutic avenues.

4.10.5 SIRT4 protein levels in normal and colorectal tumour tissue

The most surprising finding in the Histoscore analysis of SIRT4-stained tissue is the nuclear staining observed in both normal and tumour tissue. The antibody used in these studies was polyclonal and directed to C-terminal amino acids of human SIRT4, ensuring that it did not interfere with mitochondrial localisation(Abcam 2010).

Nuclear staining was not expected. This was weak and by no means ubiquitous. However, it was substantially higher than SIRT3 staining, and although the fact and relevance of SIRT3's presence in the nucleus has been the subject of controversy, substrates and physiological relevance of SIRT3 action in the nucleus are coming to light. Therefore, it is possible that this report of SIRT4 staining in the nucleus of colorectal cells may represent hitherto unsuspected activity in transcription regulation, as is seen in many of the other sirtuins.

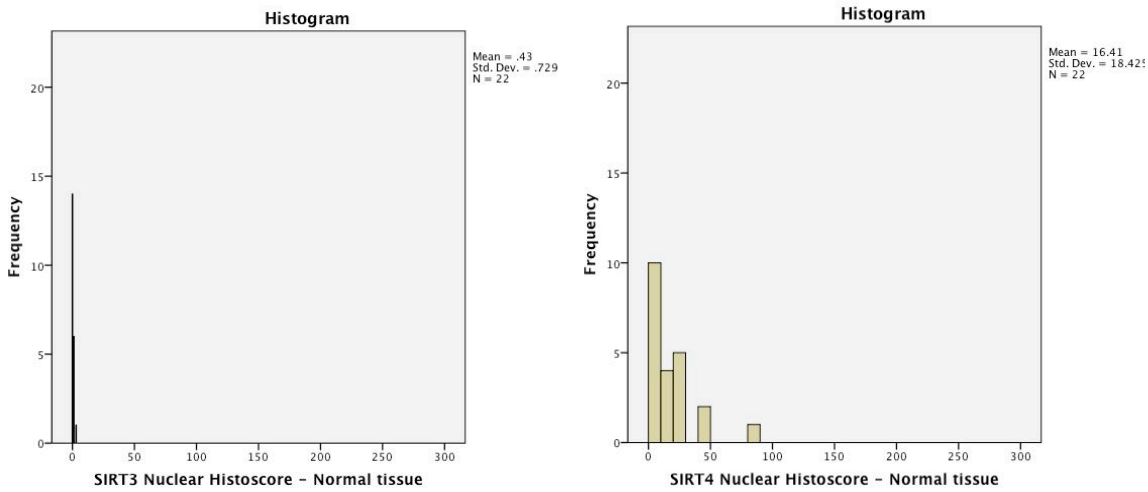

Figure 4.65 Comparison of SIRT3 and SIRT4 nuclear Histoscores in normal colorectal tissue.

SIRT4 nuclear staining was significantly reduced in tumour tissue. It is difficult to interpret this finding given that there is no accepted substrate for SIRT4 in the nucleus. Given data suggesting that SIRT4 inhibits expression of SIRT3 and SIRT1(Nasrin, Wu et al. 2010) this finding opens the possibility of direct inhibitory action of SIRT4, with a concomitant release of inhibition of SIRT3 and SIRT1 in colorectal tumours.

Comparison of cytoplasmic SIRT4 Histoscores showed a relatively strong correlation between matched normal and tumour samples, and tumours tend to show lower SIRT4 staining. Does some characteristic of malignant transformation affect SIRT4 cytoplasmic staining in a reliable manner? Given the lack of data on

SIRT4 action at all, and particularly in cancer, it is difficult to speculate. This result stands out because it is the one example of retained order in the comparison of normal and transformed colorectal epithelium. One possibility is that retained functions of SIRT4 in the cytoplasm contribute to the transformed phenotype.

SIRT4 knockdown decreases calcium-mediated induction of mitochondrial permeability transition pore opening, and increases calcium-retaining capacity of mitochondria(Verma, Shulga et al. 2013). This may be a protective strategy for tumour cells which have lost the capacity to precisely regulate calcium flux, allowing them to manage intracellular calcium leaks, and contribute to the immortality characteristic of transformed cells.

4.10.6 SIRT5 nuclear expression in normal and colorectal tumour tissue: association with age

SIRT5 expression in the nucleus has only recently been suggested and where reported is confined to subsets of cells tested(Pfister, Ma et al. 2008; Park, Chen et al. 2013). Overexpression of SIRT3 and SIRT5 in COS7 cells has shown translocation of only SIRT3 to the nucleus(Nakamura, Ogura et al. 2008). Our data do not support SIRT5 expression in normal colorectal epithelial cell nuclei as no nuclear SIRT5 staining was observed in the 20 normal samples examined in this study, but this number is probably too small to draw definitive conclusions. It is interesting that it is expressed in tumour tissue nuclei and that there is a weak association with age (next chapter), so further corroboration and information on its function will be useful.

The antibody used here recognises a peptide based on amino acids181-230 of the human SIRT5 protein, and will therefore recognise both of the isoforms transcribed from the SIRT5 gene(Abcam 2010) but will not distinguish between them.

4.10.7 SIRT5 cytoplasmic expression: reduced but no association with survival

The significant reduction in cytoplasmic expression of SIRT5 in tumour tissues with respect to normal epithelium could have widespread effects on protein

lysine succinylation on the basis of recently reported effects in mice(Park, Chen et al. 2013). They showed that 779 proteins were subject to succinylation and that a sizeable proportion of Sirt5 was located outside the mitochondria. Confirmation of similar findings in humans and in colorectal tissue rather than fibroblasts is probably the first step before extrapolating to expect a survival effect from reduction in SIRT5 levels. Equally many of the protein targets have multiple lysine succinlylation sites, so that they could integrate regulation from several different sources. This may also be why there is no significant effect on survival in this study despite the notable variation from normal.

4.10.8 SIRT6 protein levels in normal and colorectal tumour tissue

Reports on function and localisation thus far have placed SIRT6 firmly in the nucleus, with variable nucleolar access. The most recent paper, on breast cancer, has commented on SIRT6 cytoplasmic staining, which tended to associate with better prognosis (Khongkow, Olmos et al. 2013). SIRT6 staining in colorectal tissue has been reported previously although localisation of staining was not specifically mentioned (Sebastian, Zwaans et al. 2012).

In contrast to this trend in the literature, SIRT6 cytoplasmic Histoscores were generally higher (by a factor of ten) than nuclear scores in this cohort, indicating significant protein levels in cytoplasm in both normal and tumour cases relative to the nucleus. Cytoplasmic staining patterns in some cases showed specific intracytoplasmic conglomerations, although it was not possible to tell whether these were associated with intracellular organelles or possibly with containment in vesicles.

Nuclear Histoscores in tumour tissue were higher than in normal tissue. There was a similar less marked effect on comparison of matched cytoplasmic scores, which did not reach statistical significance. The unmatched comparison between the full cohorts of tumour and normal samples did show a significant difference, and although tumour cytoplasmic SIRT6 staining shows great variability, it appears to be stronger. This is in contrast to previous work on mRNA levels of SIRT6 in colorectal and breast cancer(Ashraf, Zino et al. 2006; Maxwell 2013). Differences in post-translational processing may underlie the variation in results.

Future research is likely to shed interesting light on the function and tissue distribution of SIRT6 cytoplasmic expression.

4.10.9 SIRT6 tumour Histoscore and association with survival

There was no difference in survival distribution when SIRT6 Histoscores in either the nuclear or cytoplasmic compartments were divided by quartile. Published observations on SIRT6 expression in colorectal cancer reported reduced staining in cancer specimens, with no effect on survival in node-negative patients with high SIRT6 Histoscores, but improved survival in node-positive patients (Sebastian, Zwaans et al. 2012). Similar subgroup analysis was not performed on this cohort.

In breast cancer(Khongkow, Olmos et al. 2013), high nuclear SIRT6 was associated with poorer survival. Although SIRT6 cytoplasmic staining was noted in the tumour samples stained, it was not clear how staining patterns compare to those in normal tissue.

4.10.10 SIRT7 levels in normal and colorectal tumour tissue

The antibody used in these experiments was raised against amino acids 35-51 and 361-377 of the 400 amino acid SIRT7 protein and therefore recognises both N- and C-terminal sequences (Abcam 2010). The C-terminus contains the nucleolar localisation sequence while the nuclear localisation sequence is positioned near the N-terminus. This antibody does not distinguish between the 47.5 kDa and 45kDa forms of SIRT7. Since these differ due to post-translational processing, it is not known whether it identifies both forms at all or with the same affinity.

The only previous publication to mention SIRT7 staining in human colorectal tissue reported heavy cytoplasmic staining in normal colorectal tissue, but no nuclear staining (Kiran, Chatterjee et al. 2013). In contrast, nuclear staining was noted in both normal and tumour tissue in the TMA examined here, and was comparable in both tissue groups.

Cytoplasmic staining measured by Histoscore was much higher than nuclear staining, in both normal and tumour tissue, in this dataset. The unmatched

comparison showed significantly lower cytoplasmic Histoscore in tumour tissue with respect to normal tissue. This is in keeping with mRNA studies which showed lower levels of SIRT7 mRNA in colorectal tumour tissue in comparison to matched normal samples, and with the previous study on colorectal tissue alluded to above. Further information on the function of this highly prevalent protein in the cytoplasm will be enlightening.

4.10.11 SIRT7 tumour Histoscore and association with survival

There is no difference between survival distributions for SIRT7 nuclear Histoscores divided in quartiles on Kaplan-Meier analysis. On Kaplan-Meier analysis of SIRT7 cytoplasmic Histoscore divided in quartiles, there was a significant difference in survival distributions on log-rank test, but reduced survival was associated with second quartile and highest quartile SIRT7 cytoplasmic Histoscore. There was no incremental association with SIRT7 Histoscore quartiles, or association with highest and lowest quartiles of SIRT7 Histoscore as seen with SIRT2 nuclear Histoscores. On univariate survival analysis, SIRT7 Histoscore divided in quartiles had no significant effect on survival. It was not therefore included in multivariate regression analysis. It is unlikely that the significant difference in survival distributions noted in Kaplan-Meier analysis translates into a true effect on survival from colorectal cancer.

There has only been one previous report on a survival effect of SIRT7 protein expression in cancer: in 39 cases of head and neck cancer, protein expression was nuclear rather than cytoplasmic and lower than in normal tissue (Lai, Lin et al. 2013). Lai et al reported no association with survival. These results are in keeping with the findings here in colorectal cancer.

5 Sirtuin Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between clinicopathological factors and Histoscores for each combination of sirtuin and subcellular location in tumour samples were examined; the rationale for these analyses was explained in the Experimental Methods chapter. There were insufficient numbers of normal samples in the TMA used for meaningful analysis of associations with clinicopathological factors. Data on associations between 23 clinicopathological variables and sirtuin Histoscores in nucleus and cytoplasm in colorectal tumour tissue are presented below. $p<0.01$ was accepted as the level of statistical significance; associations with p-value >0.01 are lowlighted in grey text in summary results tables.

5.1 Clinical and Pathological Characteristics of the Patient Cohort

Clinical and pathological characteristics of this patient cohort (n=272) are summarised in the table overleaf.

Sex	M	147	**Dukes' stage**	A	20
	F	125		B	132
	Total	*272*		C	119
Age	<65	97		D	1
	65<X<75	89		*Total*	*272*
	>75	86	**T stage**	1	9
	Total	*272*		2	20
BMI	<20	15		3	162
	20-25	81		4	81
	25.1-30	71		*Total*	*272*
	>30	25	**N stage**	0	152
	Total	*192*		1	91
Albumin	<35	230		2	29
	>35	42		*Total*	*272*
	Total	272	**Differentiation**	Poor	33
CRP	>10	123		Well/Mod	239
	<10	149		*Total*	*272*
	Total	*272*	**Peritoneum involved**	Y	80
mGPS	0	149		N	192
	1	91		*Total*	*272*
	2	32	**Margin involved**	Y	21
	Total	*272*		N	251
Anaemia	None	116		*Total*	*272*
	Mild	65	**Venous invasion**	Y	99
	Severe	55		N	173
	Total	*236*		*Total*	*272*
Smoker	N	98	**Tumour perforation**	Y	10
	Y	27		N	262
	Total	*125*		*Total*	*272*
Elective presentation	Y	254	**High risk Petersen Index**	Y	52
	N	18		N	220
	Total	*272*		*Total*	*272*
Tumour location	Colon	183	**PTI**	Y	53
	Rectum	89		N	195
	Total	*272*		*Total*	*248*

Table 5.1 Clinicopathological characteristics of the patient cohort who contributed tissue to the TMA used for sirtuin Histoscore analysis.

5.2 SIRT2 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between SIRT2 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the following tables and significant associations are subsequently discussed in more detail. Associations with extreme quartiles of SIRT2 nuclear Histoscores were assessed in view of the relationship between extreme quartiles of SIRT2 nuclear Histoscore and survival described in the previous chapter.

SIRT2		Nucleus	Nucleus: extreme quartiles	Cytoplasm
Demographic variables	Age	Pearson Chi square, p=0.606	Extreme quartile Histoscores were associated with extremes of age. Pearson Chi square, p=0.045, Linear-by-linear, p=0.940	Pearson Chi square, p=0.513
	Sex	Pearson Chi square, p=0.806	Pearson Chi square, p=0.623	Pearson Chi square, p=0.049
	BMI	Pearson Chi square, p=0.654	Pearson Chi square, p=0.518	Low Histoscores associated with increased BMI. Pearson Chi square, p=0.047, linear-by-linear, p=0.031
Inflammatory markers	WCC	High Histoscores were associated with high WCC. Kruskal-Wallis test, p=0.0225	Pearson Chi square, p=0.069	Pearson Chi square, p=0.918
	CRP	Highest quartile Histoscores were associated with high CRP. Kruskal-Wallis test, p=0.0038	Extreme quartile Histoscores were associated with high CRP. Mann-Whitney U test, p=0.001	Pearson Chi square, p=0.622
	Albumin	Lowest quartile Histoscores were associated with low albumin. Pearson Chi square, p=0.027	Extreme quartile Histoscores were associated with high albumin. Pearson Chi square, p=0.009	Lower albumin was associated with lowest quartile SIRT2 cytoplasmic staining (Mann-Whitney U test, p=0.0068).
	mGPS	Highest quartile Histoscore was associated with high mGPS. Pearson Chi square, p=0.028, linear-by-linear, p=0.010	Extreme quartile Histoscores were associated with high mGPS. Pearson Chi square, p=0.001, linear-by-linear, p=0.0018	Pearson Chi square, p=0.866

Table 5.2 Associations between SIRT2 nuclear and cytoplasmic Histoscores and demographic variables, and classical markers of inflammation. Relationships with extreme quartiles of nuclear Histoscores were assessed in view of the relationship between extreme quartiles of SIRT2 nuclear Histoscore and survival described in the previous chapter. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

Table 5.3 Associations between SIRT2 nuclear and cytoplasmic Histoscores and atypical markers of inflammation, mode of presentation, tumour location, measures of cancer stage and Charlson Comorbidity Index. Relationships with extreme quartiles of nuclear Histoscores were assessed in view of the relationship between extreme quartiles of SIRT2 nuclear Histoscore and survival described in the relevant chapter. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT2		Nucleus	Nucleus: extreme quartiles	Cytoplasm
Atypical markers of inflammation	**Hb**	Highest quartile was associated with severe anaemia. Pearson Chi square, p=0.027, linear-by-linear, p=0.096	Pearson Chi square, p=0.380	Pearson Chi square, p=0.829
	Smoking status	Pearson Chi square, p=0.503	Pearson Chi square, p=0.104	Pearson Chi square, p=0.233
Presentation		Lower Histoscore was associated with elective presentation, Pearson Chi square, p=0.014	Extreme quartile Histoscores were associated with elective presentation, Pearson Chi square, p=0.015	Pearson Chi square, p=0.625
Charlson Comorbidity Index		Pearson Chi square, p=0.092	Pearson Chi square, p=0.310	Pearson Chi square, p=0.154
Tumour location		Pearson Chi square, p=0.793	Pearson Chi square, p=0.600	Pearson Chi square, p=0.793
Measures of cancer staging	**Dukes' stage**	Low Histoscore was associated with lower Dukes' stage. Pearson Chi square, p=0.058, Linear-by-linear, p=0.040	Pearson Chi square, p=0.259	Pearson Chi square, p=0.638
	T stage	Higher Histoscore was associated with higher T stage. Pearson Chi square, p=0.045, linear-by-linear, p=0.018	Extreme Histoscore was associated with higher T stage. Pearson Chi square, p=0.150, linear-by-linear association, p=0.0430	Pearson Chi square, p=0.470
	N stage	Highest quartile Histoscore was associated with higher N stage. Pearson Chi square, p=0.032, linear-by-linear, p=0.013	Pearson Chi square, p=0.159	Pearson Chi square, p=0.798
	M stage	Pearson Chi square, p=0.316	Pearson Chi square, p=0.316	Pearson Chi square, p=0.316

SIRT2		Nucleus	Nucleus: extreme quartiles	Cytoplasm
Pathological prognostic markers	Differentiation	Higher Histoscores were associated with poor differentiation. Mann-Whitney U test, p=0.0007	Pearson Chi square, p=0.132	Pearson Chi square, p=0.706
	Margin involvement	Pearson Chi square, p=0.642	Pearson Chi square, p=0.163	Highest quartile was associated with margin involvement. Pearson Chi square, p=0.043
	Peritoneal involvement	Pearson Chi square, p=0.178	Pearson Chi square, p=0.059	Lowest quartile Histosore was associated with peritoneal involvement. Pearson Chi square, p=0.017
	Tumour perforation	Pearson Chi square, p=0.197	Pearson Chi square, p=0.197	Pearson Chi square, p=1.000
	Venous invasion	Highest quartile Histoscore was associated with venous invasion. Pearson Chi square, p=0.0091	Extreme quartile Histoscores were associated with venous invasion. Pearson Chi square, p=0.022	Pearson Chi square, p=0.309
	High risk Petersen index	Highest quartile Histoscore was associated with high risk PI. Mann-Whitney U test, p=0.0378	Extreme quartile Histoscores were associated with high risk PI. Pearson Chi square, p=0.003	Pearson Chi square, p=0.640
Peritumoural Inflammation		High Histoscore was associated with absence of PTI. Mann-Whitney U test, p=0.0483	Pearson Chi square, p=0.696	Pearson Chi square, p=0.377

Table 5.4 Associations between SIRT2 nuclear and cytoplasmic Histoscores and histopathological markers of prognostic significance in colorectal cancer. Relationships with extreme quartiles of nuclear Histoscores were assessed in view of the relationship between extreme quartiles of SIRT2 nuclear Histoscore and survival described in the relevant chapter. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

5.2.1 Association with inflammatory markers

5.2.1.1 Association with serum C-reactive protein

Extremes of SIRT2 staining in the nucleus (highest and lowest quartiles) were associated with higher serum CRP. Kruskal-Wallis test indicated this pattern as shown in the boxplots below (p=0.0038), and the relationship was stronger when SIRT2 nuclear Histoscores were categorised into 'extreme' (lowest and highest quartile) and middle quartiles (Mann-Whitney U test, p=0.001).

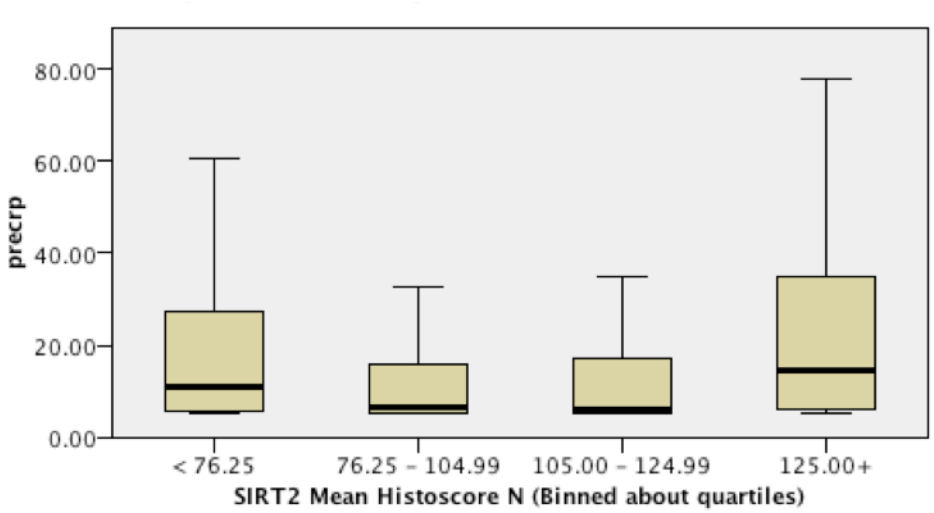

Figure 5.1 Boxplots showing distribution of preoperative serum CRP categorised according to quartiles of SIRT2 nuclear Histoscores. Highest and lowest quartile SIRT2 nuclear Histoscores were associated with higher CRP (Mann-Whitney U test, p=0.001).

5.2.1.2 Association with serum albumin

Low albumin was associated with extremes of SIRT2 nuclear staining (Chi square test, p=0.009).

		SIRT2 Nuclear Histoscore		
		Highest or lowest quartile	Middle 2 quartiles	Total
Albumin	>35	106	121	227
	<35	27	12	39
Total		133	133	266

Table 5.5 Crosstabulation of high and low albumin (split about 35, the dividing point used in the modified Glasgow Prognostic Score) and SIRT2 nuclear Histoscores, divided into 'extremes' (highest and lowest quartiles, and middle two quartiles). Chi square analysis showed significant association between extreme values of SIRT2 nuclear Histoscores and low albumin (p=0.009).

Lower albumin was associated with lowest quartile SIRT2 cytoplasmic staining (Mann-Whitney U test, p=0.0068).

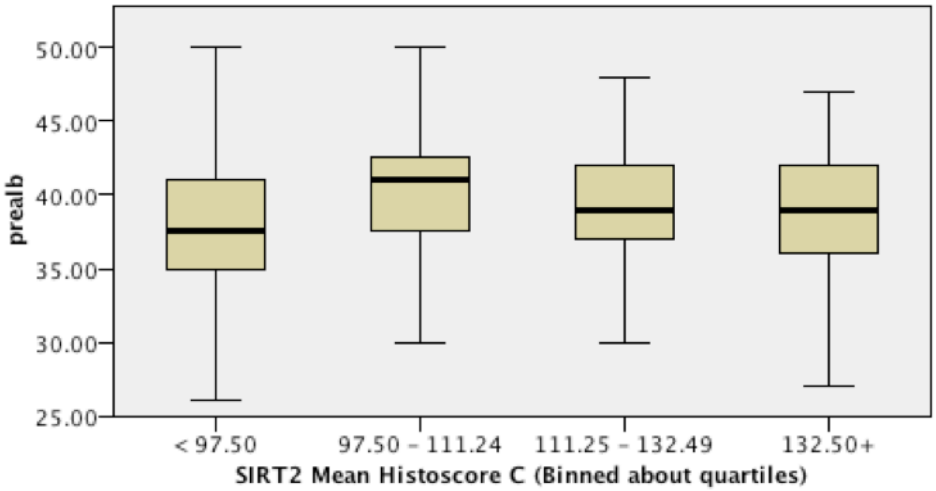

Figure 5.2 Boxplots showing preoperative albumin categorised by SIRT2 nuclear Histoscore split in quartiles (Kruskal-Wallis test, p=0.0275). Mann-Whitney U test showed that low albumin is associated with lowest quartile SIRT2 nuclear Histoscore (p=0.0068).

5.2.1.3 Association with modified Glasgow Prognostic Score

High modified Glasgow Prognostic Score (mGPS) was associated with highest quartile SIRT2 nuclear staining, (Pearson Chi square, p=0.028, linear-by-linear, p=0.010).

		SIRT2 Nuclear Histoscore		
		Highest quartile	3 lowest quartiles	Total
mGPS	0	118	28	146
	1	61	29	90
	2	19	11	30
Total		198	68	266

Table 5.6 Crosstabulation of mGPS with SIRT2 nuclear Histoscores by highest quartile and lower 3 quartiles. Highest quartile Histoscore is associated with high mGPS. Pearson Chi square, p=0.028, linear-by-linear, p=0.010.

High mGPS (more inflammation) was more strongly associated with extremes of SIRT2 nuclear staining (Pearson Chi square, p=0.001, linear-by-linear association, p=0.00018).

		SIRT2 Nuclear Histoscore		
		Extreme quartiles	Middle 2 quartiles	Total
mGPS	0	60	86	146
	1	50	40	90
	2	23	7	30
Total		133	133	266

Table 5.7 Crosstabulation of mGPS with SIRT2 nuclear Histoscores categorised in extreme quartiles, and middle quartiles showed a stronger relationship than simply with SIRT2 nuclear Histoscores categorised in quartiles (Pearson Chi square, p=0.001, linear-by-linear association, p=0.00018.

5.2.2 Association with histological markers of prognostic significance

5.2.2.1 Associations with histological factors of prognostic significance

Higher SIRT2 nuclear staining was associated with poorly differentiated tumours (Mann-Whitney U test, p=0.0007).

Highest quartile SIRT2 nuclear staining was associated with venous invasion (Chi square test, p=0.0091).

		SIRT2 Nuclear Histoscore		
		3 lower quartiles	Highest quartile	Total
Venous invasion	N	134	34	168
	Y	64	34	98
Total		198	68	266

Table 5.8 Crosstabulation of venous invasion and SIRT2 nuclear Histoscore showed significant association between lowest quartile SIRT2 nuclear Histoscore and venous invasion (Chi square analysis, p=0.0091).

5.3 SIRT3 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between SIRT3 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the following tables and significant associations are subsequently discussed in more detail.

SIRT3		Nucleus	Cytoplasm
Demographic variables	**Age**	Pearson Chi square, p=0.307	Higher Histoscore was associated with older age. Mann-Whitney U test, p=0.0185
	Sex	Pearson Chi square, p=0.553	Pearson Chi square, p=0.109
	BMI	Pearson Chi square, p=0.815	Pearson Chi square, p=0.932
Inflammatory markers	**WCC**	Higher Histoscores were associated with high WCC. Pearson Chi square, p=0.092, linear-by-linear, p=0.029	Lower Histoscores were associated with high WCC. Mann-Whitney U test, p=0.0059
	CRP	Pearson Chi square, p=0.692	Low Histoscore was associated with high CRP. Mann-Whitney U test, p=0.0009
	Albumin	Highest quartile SIRT3 nuclear Histoscores were associated with higher preoperative serum albumin, Mann-Whitney U test, p=0.012.	Lowest quartile Histoscore was associated with lower albumin. Mann-Whitney U test, p=0.0438
	mGPS	Pearson Chi square, p=0.499	Higher Histoscores were associated with low mGPS. Pearson Chi square, p=0.062, linear-by-linear, p=0.026
Atypical markers of inflammation	**Hb**	Pearson Chi square, p=0.989	Lower Histoscores were associated with anaemia. Mann-Whitney U test, p=0.00029
	Smoking status	High Histoscore was associated with smoking status. Pearson Chi square, p=0.036	Pearson Chi square, p=0.418
Presentation		Presence of nuclear staining was associated with emergency presentation. Mann-Whitney U test, p=0.038	Pearson Chi square, p=0.452
Charlson Comorbidity Index		Pearson Chi square, p=0.747	Pearson Chi square, p=0.206
Tumour location		Pearson Chi square, p=0.825	Pearson Chi square, p=0.151

Table 5.9 Associations between SIRT3 nuclear and cytoplasmic Histoscores and demographic variables, classical and atypical markers of inflammation, comorbidity, presentation and tumour location. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT3		Nucleus	Cytoplasm
Measures of cancer staging	Dukes' stage	Pearson Chi square, p=0.488	Pearson Chi square, p=0.743
	T stage	Pearson Chi square, p=0.704	Low Histoscore was associated with higher T stage. Pearson Chi square, p=0.064, linear-by-linear, p=0.034
	N stage	Pearson Chi square, p=0.614	Pearson Chi square, p=0.806
	M stage	Pearson Chi square, p=0.309	Pearson Chi square, p=0.316
Pathological prognostic markers	Differentiation	Nuclear staining was associated with poor differentiation. Pearson Chi square, p=0.013	Poor differentiation was associated with lower Histoscore. Mann-Whitney U test, p=0.0055
	Margin involvement	Pearson Chi square, p=0.593	Pearson Chi square, p=0.163
	Peritoneal involvement	Pearson Chi square, p=0.703	Pearson Chi square, p=0.106
	Tumour perforation	Pearson Chi square, p=0.099	Pearson Chi square, p=0.309
	Venous invasion	Nuclear staining was associated with venous invasion, Pearson Chi square, p=0.005	Pearson Chi square, p=0.446
	High risk Petersen index	Pearson Chi square, p=0.410	Pearson Chi square, p=0.117
Peritumoural Inflammation		Pearson Chi square, p=0.525	Lowest quartile Histoscore was associated with PTI. Pearson Chi square, p=0.033

Table 5.10 Associations between SIRT3 nuclear and cytoplasmic Histoscores and accepted factors of prognostic significance in colorectal cancer (cancer staging systems and histopathological markers). Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

5.3.1 Association with inflammatory markers

5.3.1.1 Association with white cell count

SIRT3 cytoplasmic staining above the median was associated with lower white cell count (Mann-Whitney U test, p=0.0059).

5.3.1.2 Association with serum C-reactive protein (CRP)

SIRT3 cytoplasmic Histoscores above the median were associated with lower preoperative serum CRP (Mann-Whitney U test, p=0.0009). There is a parallel between this association and the relationship with white cell count.

5.3.2 Association with atypical inflammatory markers

5.3.2.1 Association with haemoglobin

Higher haemoglobin was associated with SIRT3 cytoplasmic Histoscores above the median (Mann-Whitney U test, p=0.00029).

5.3.3 Associations with histological factors of prognostic significance in colorectal cancer

Poorly differentiated tumours were more likely to have low cytoplasmic SIRT3 staining (Mann-Whitney U test, p=0.0055).

Presence of SIRT3 nuclear staining was associated with peritumoural venous invasion (a positive prognostic factor) (Pearson Chi square, p=0.005).

		SIRT3 Nuclear Histoscore		
		Absent	Present	Total
Venous invasion	N	70	98	168
	Y	24	74	98
Total		94	172	266

Table 5.11 Crosstabulation of venous invasion and presence of SIRT3 nuclear staining. There was an association between presence of nuclear staining and venous invasion (Pearson Chi square, p=0.005).

5.4 SIRT4 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between SIRT4 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the table above and significant associations are then discussed in more detail.

Table 5.12 (following page) Associations between SIRT4 nuclear and cytoplasmic Histoscores and demographic variables, markers of inflammation and accepted factors of prognostic significance in colorectal cancer. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT4		Nucleus	Cytoplasm
Demographic variables	**Age**	Pearson Chi square, p=0.879	Pearson Chi square, p=0.568
	Sex	Pearson Chi square, p=0.275	Pearson Chi square, p=0.781
	BMI	Pearson Chi square, p=0.294	Pearson Chi square, p=0.936
Inflammatory markers	**WCC**	Pearson Chi square, p=0.203	Lowest quartile Histoscore was associated with normal range WCC. Kruskal-Wallis test, p=0.0280
	CRP	Pearson Chi square, p=0.342	Lower quartile Histoscores were associated with higher CRP. Kruskal-Wallis test, p=0.00015
	Albumin	Pearson Chi square, p=0.341	Pearson Chi square, p=0.838
	mGPS	Pearson Chi square, p=0.635	Higher Histoscores were associated with low mGPS. Kruskal-Wallis test, p=0.0190
Atypical markers of inflammation	**Hb**	Pearson Chi square, p=0.333	Pearson Chi square, p=0.675
	Smoking status	Pearson Chi square, p=0.167	Pearson Chi square, p=0.253
	Presentation	Pearson Chi square, p=0.135	Higher Histoscore was associated with elective presentation, Pearson Chi square, p=0.020
Charlson Comorbidity Index		Pearson Chi square, p=0.300	Higher Histoscore was associated with lower CCI. Pearson Chi square, p=0.009, linear-by-linear, p=0.261
Tumour location		Pearson Chi square, p=0.906	Pearson Chi square, p=0.832
Measures of cancer staging	**Dukes' stage**	Pearson Chi square, p=0.537	Pearson Chi square, p=0.698
	T stage	Pearson Chi square, p=0.450	Pearson Chi square, p=0.721
	N stage	Pearson Chi square, p=0.259	Pearson Chi square, p=0.784
	M stage	Pearson Chi square, p=0.343	Pearson Chi square, p=0.307
Pathological prognostic markers	**Differentiation**	Pearson Chi square, p=0.968	Pearson Chi square, p=0.214
	Margin involvement	Pearson Chi square, p=0.256	Pearson Chi square, p=0.705
	Peritoneal involvement	Pearson Chi square, p=0.207	Pearson Chi square, p=0.386
	Tumour perforation	Pearson Chi square, p=0.610	Pearson Chi square, p=0.080
	Venous invasion	Nuclear staining was associated with venous invasion. Mann-Whitney U test, p=0.0181	Highest quartile Histoscore was associated with venous invasion. Pearson Chi square, p=0.030
	High risk Petersen index	Pearson Chi square, p=0.168	Pearson Chi square, p=0.646
Peritumoural Inflammation		Pearson Chi square, p=0.110	Highest quartile Histoscore was associated with absence of PTI. Pearson Chi square, p=0.029

5.4.1 Association with inflammatory markers

5.4.1.1 Association with serum C-reactive protein (CRP)

Higher CRP was associated with SIRT4 cytoplasmic Histoscore in the lower quartiles (Kruskal-Wallis test, p=0.00015).

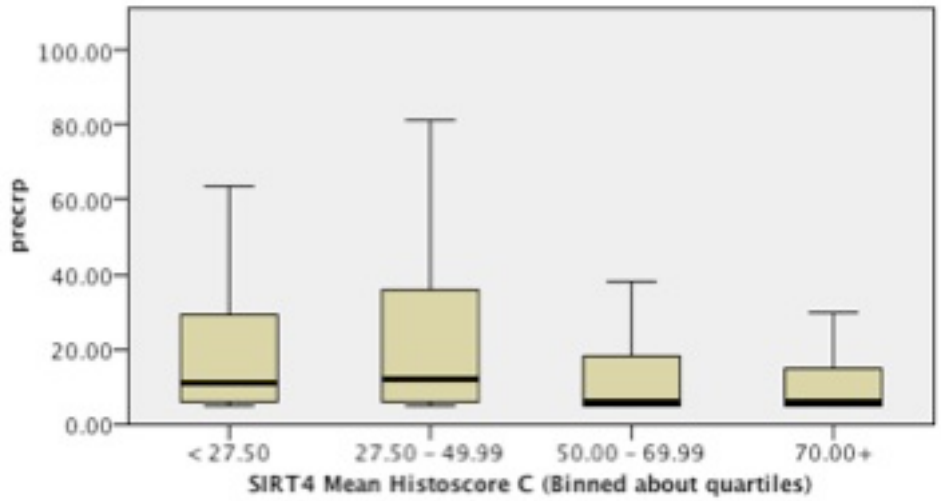

Figure 5.3 Boxplots of CRP categorised according to SIRT4 cytoplasmic Histoscore quartiles. There was a significant difference in the distribution of CRP grouped according to SIRT4 cytoplasmic Histoscore in quartiles. Higher CRP was associated with lower cytoplasmic SIRT4 Histoscores (Kruskal-Wallis test, p=0.00015).

5.5 SIRT5 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

SIRT5 nuclear staining was infrequent and very weak and was therefore categorised as absent or present rather than in quartiles or about the median, as was the categorisation used for the other sirtuins and SIRT5 cytoplasmic staining. Associations between SIRT5 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the following tables and statistically significant associations are subsequently discussed in more detail.

Table 5.13 (following page) Associations between SIRT5 nuclear and cytoplasmic Histoscores and demographic variables, markers of inflammation and accepted factors of prognostic significance in colorectal cancer. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT5		Nucleus	Cytoplasm
Demographic variables	**Age**	Nuclear staining was associated with younger age. Kruskal-Wallis test, p=0.044.	Pearson Chi square, p=0.133
	Sex	Pearson Chi square, p=0.510	Pearson Chi square, p=0.858
	BMI	Pearson Chi square, p=0.407	Pearson Chi square, p=0.287
Inflammatory markers	**WCC**	Pearson Chi square, p=0.617	Lowest quartile Histoscore was associated with higher WCC. Mann-Whitney U test, p=0.0061
	CRP	Pearson Chi square, p=0.201	Lowest quartile was associated with high CRP. Mann-Whitney U test, p=0.0095
	Albumin	Nuclear staining was associated with higher albumin. Mann-Whitney U test, p=0.0234	Lowest quartile Histoscore was associated with lower serum albumin. Mann-Whitney U test, p=0.017
	mGPS	Pearson Chi square, p=0.435	Highest quartile was associated with low mGPS. Pearson Chi square, p=0.011, linear-by-linear, p=0.006
Atypical markers of inflammation	**Hb**	Pearson Chi square, p=0.623	Pearson Chi square, p=0.968
	Smoking status	Pearson Chi square, p=0.708	Pearson Chi square, p=0.703
	Presentation	Pearson Chi square, p=0.116	Pearson Chi square, p=0.443
Charlson Comorbidity Index		Pearson Chi square, p=0.756	Pearson Chi square, p=0.114
Tumour location		Pearson Chi square, p=0.325	Pearson Chi square, p=0.762
Measures of cancer staging	**Dukes' stage**	Pearson Chi square, p=0.604	Pearson Chi square, p=0.410
	T stage	Pearson Chi square, p=0.640	Pearson Chi square, p=0.775
	N stage	Pearson Chi square, p=0.498	Pearson Chi square, p=0.201
	M stage	Pearson Chi square, p=0.712	Pearson Chi square, p=0.318
Pathological prognostic markers	**Differentiation**	Pearson Chi square, p=0.550	Poor differentiation was associated with lowest quartile Histoscore. Pearson Chi square, p=0.017
	Margin involvement	Pearson Chi square, p=0.718	Higher Histoscore was associated with margin involvement. Mann-Whitney U test, p=0.0196
	Peritoneal involvement	Pearson Chi square, p=0.865	Pearson Chi square, p=0.446
	Tumour perforation	Pearson Chi square, p=0.336	Tumour perforation was associated with lower Histoscore. Mann-Whitney U test, p=0.0372
	Venous invasion	Pearson Chi square, p=0.221	Pearson Chi square, p=0.401
	High risk Petersen index	Pearson Chi square, p=0.670	Pearson Chi square, p=0.454
Peritumoural Inflammation		Pearson Chi square, p=0.311	Pearson Chi square, p=0.930

5.5.1 Association with inflammatory markers

5.5.1.1 Association with white cell count

Lowest quartile SIRT5 cytoplasmic Histoscore was associated with higher white cell count (Mann-Whitney U test, p=0.0061).

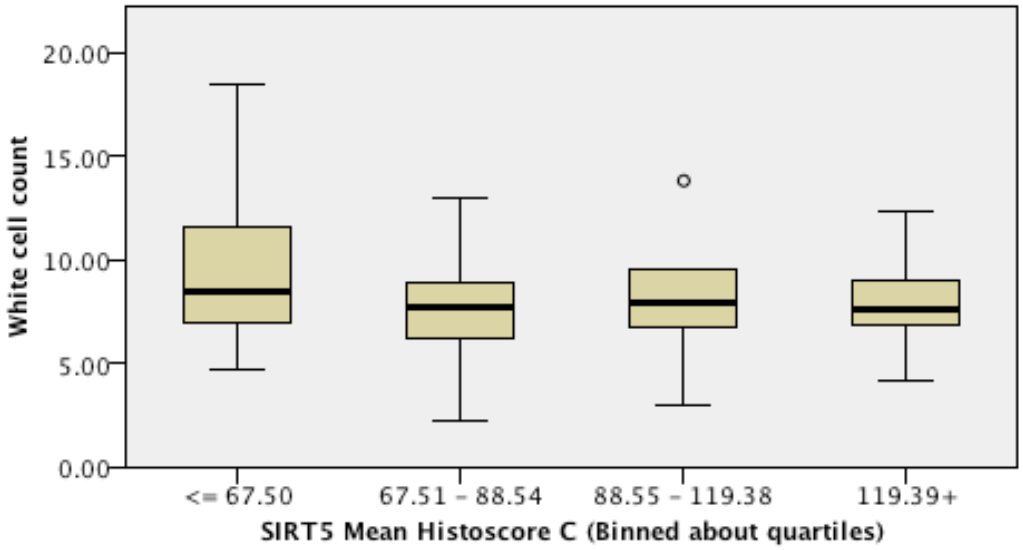

Figure 5.4 Boxplots showing distribution of white cell count categorised by SIRT5 cytoplasmic Histoscore in quartiles. Lowest quartile SIRT5 cytoplasmic Histoscore was associated with higher white cell count (Mann-Whitney U test, p=0.0061).

5.5.1.2 Association with serum C-reactive protein (CRP)

Lowest quartile SIRT5 cytoplasmic staining was associated with higher serum CRP (Mann-Whitney U test, p=0.0095).

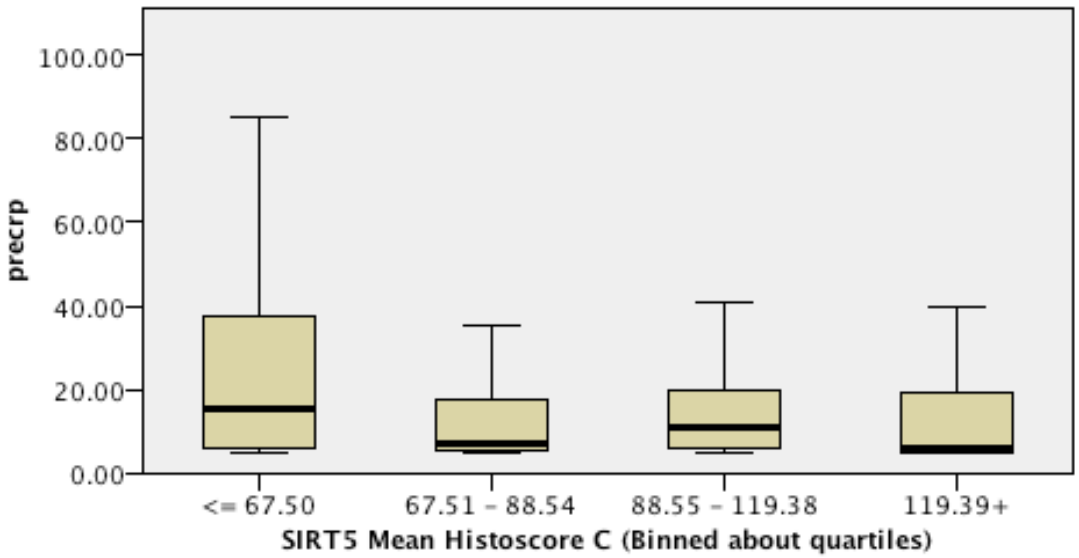

Figure 5.5 Boxplots showing preoperative serum CRP categorised by SIRT5 cytoplasmic Histoscore in quartiles. Lowest quartile SIRT5 cytoplasmic Histoscore was associated with higher CRP (Mann-Whitney U test, p=0.0095).

5.5.1.3 Association with modified Glasgow Prognostic Score (mGPS)

Highest quartile SIRT5 cytoplasmic staining is associated with mGPS (Chi square test, linear by linear association, p=0.0248).

		SIRT5 Cytoplasmic Histoscore		
		Lower 3 quartiles	Highest quartile	Total
mGPS	0	101	44	145
	1	70	20	90
	2	29	3	32
Total		200	67	267

Table 5.14 Crosstabulation of mGPS with SIRT5 cytoplasmic Histoscores categorised highest and lower three quartiles. Low mGPS was associated with highest quartile cytoplasmic Histoscore (Pearson Chi square, p=0.011, linear-by-linear, p=0.006).

5.6 SIRT6 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between SIRT6 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the following tables and significant associations are subsequently discussed in more detail.

SIRT6		Nucleus	Cytoplasm
Demographic variables	Age	Pearson Chi square, p=0.929	Lowest quartile was associated with lower age. Pearson Chi square, p=0.027, linear-by-linear, p=0.096
	Sex	Pearson Chi square, p=0.027	Female sex was associated with lower Histoscore. Mann-Whitney U test, p=0.0357
	BMI	Highest quartile Histoscore was associated with higher BMI. Mann-Whitney U test, p=0.0005	Pearson Chi square, p=0.287
Inflammatory markers	WCC	Lowest quartile Histoscore was associated with lower WCC. Mann-Whitney U test, p=0.0331	Histoscores above the median were associated with lower WCC. Mann-Whitney U test, p=0.0009
	CRP	Histoscores above the median were associated with low CRP. Mann-Whitney U test, p=0.00013	Highest quartile Histoscore was associated with low CRP. Mann-Whitney U test, p=0.00003
	Albumin	Pearson Chi square, p=0.431	Highest quartile SIRT6 cytoplasmic Histoscore was associated with higher serum albumin. Mann-Whitney U test, p=0.0051
	mGPS	Higher Histoscore was associated with high mGPS. Kruskal-Wallis test, p=0.0008	Lower Histoscore was associated with high mGPS. Kruskal-Wallis test, p=0.0054
Atypical markers of inflammation	Hb	Pearson Chi square, p=0.534	Highest quartile Histoscore was associated with higher haemoglobin. Mann-Whitney U test, p=0.0036
	Smoking status	Pearson Chi square, p=0.644	Pearson Chi square, p=0.369
Presentation		Pearson Chi square, p=0.492	Pearson Chi square, p=0.452
Charlson Comorbidity Index		Pearson Chi square, p=0.119	Pearson Chi square, p=0.630
Tumour location		Pearson Chi square, p=0.762	Pearson Chi square, p=0.509

Table 5.15 Associations between SIRT6 nuclear and cytoplasmic Histoscores and demographic variables, classical and atypical markers of inflammation, presentation, comorbidity and tumour location. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT6		Nucleus	Cytoplasm
Measures of cancer staging	Dukes' stage	Lowest quartile Histoscore was associated with lower Dukes' stage. Pearson Chi square, p=0.025, linear-by-linear, p=0.049	Pearson Chi square, p=0.582
	T stage	Pearson Chi square, p=0.679	Pearson Chi square, p=0.766
	N stage	Lowest quartile Histoscore was associated with lower N stage. Pearson Chi square, p=0.061, linear-by-linear, p=0.036	Pearson Chi square, p=0.641
	M stage	Pearson Chi square, p=0.309	Pearson Chi square, p=0.316
Pathological prognostic markers	Differentiation	Pearson Chi square, p=0.114	Poor differentiation was associated with lower Histoscore. Mann-Whitney U test, p=0.0008
	Margin involvement	Pearson Chi square, p=0.287	Low Histoscore was associated with margin involvement. Mann-Whitney U test, p=0.0268
	Peritoneal involvement	Pearson Chi square, p=0.571	Pearson Chi square, p=0.346
	Tumour perforation	Pearson Chi square, p=0.331	Pearson Chi square, p=0.090
	Venous invasion	Highest quartile Histoscore was associated with venous invasion. Pearson Chi square, p=0.010	Pearson Chi square, p=0.307
	High risk Petersen index	Pearson Chi square, p=0.972	Lowest quartile Histoscore was associated with high risk PI. Pearson Chi square, p=0.008
Peritumoural Inflammation		Pearson Chi square, p=0.616	Pearson Chi square, p=0.478

Table 5.16 Associations between SIRT6 nuclear and cytoplasmic Histoscores and accepted factors of prognostic significance in colorectal cancer (cancer staging systems and histopathological markers). Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

5.6.1 Association with demographic factors.

5.6.1.1 Associations with BMI

Highest quartile SIRT6 nuclear Histoscore was associated with higher BMI (Mann-Whitney U test, p=0.0005).

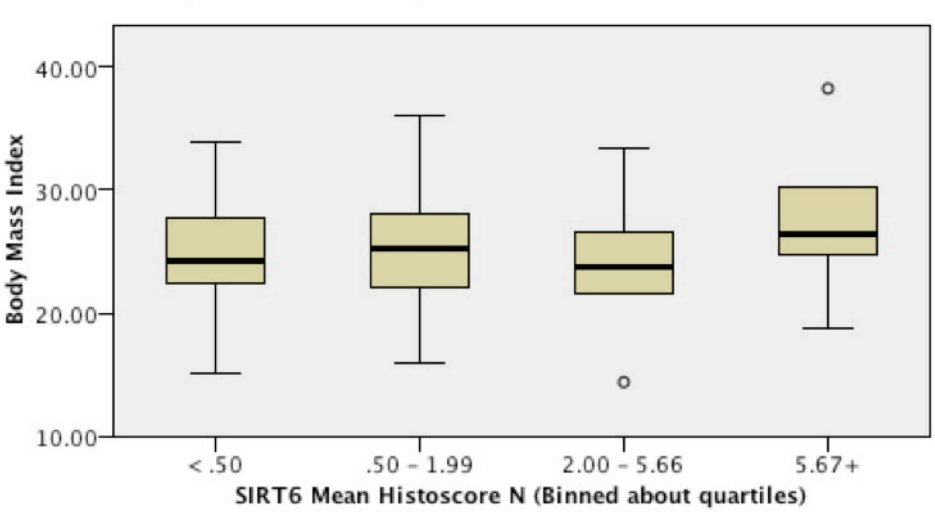

Figure 5.6 Boxplots showing distribution of BMI categorised by quartile of SIRT6 nuclear Histoscore. Highest quartile SIRT6 nuclear Histoscore was associated with higher BMI (Mann-Whitney U test, p=0.0005).

5.6.2 Association with inflammatory markers

5.6.2.1 Association with white cell count

Lower white cell counts were associated with SIRT6 cytoplasmic Histoscore above the median (Mann-Whitney U test, p=0.0009).

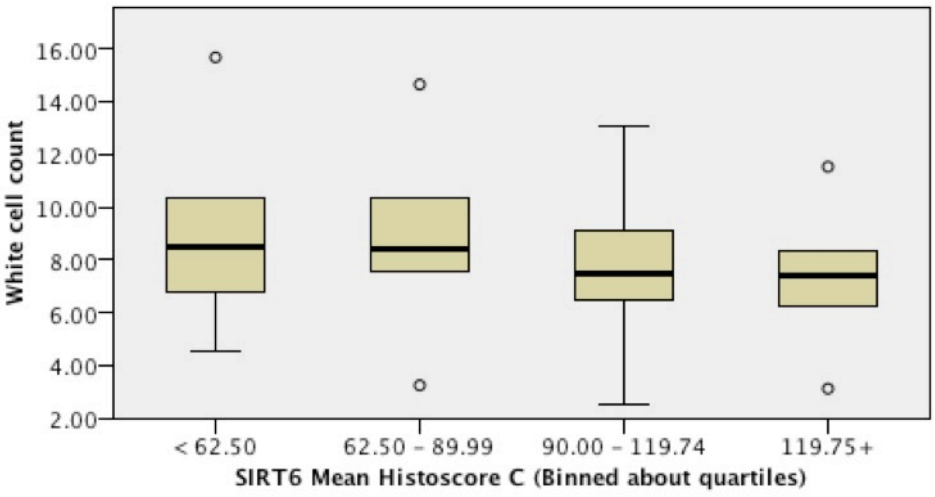

Figure 5.7 Boxplots showing distribution of white cell count by quartiles of SIRT6 cytoplasmic Histoscore. SIRT6 cytoplasmic Histoscore above the median was associated with lower white cell count (Mann-Whitney U test, p=0.0009).

5.6.2.2 Association with serum C-reactive protein (CRP)

SIRT6 nuclear Histoscores above the median were associated with higher CRP (Mann-Whitney U test, p=0.00013).

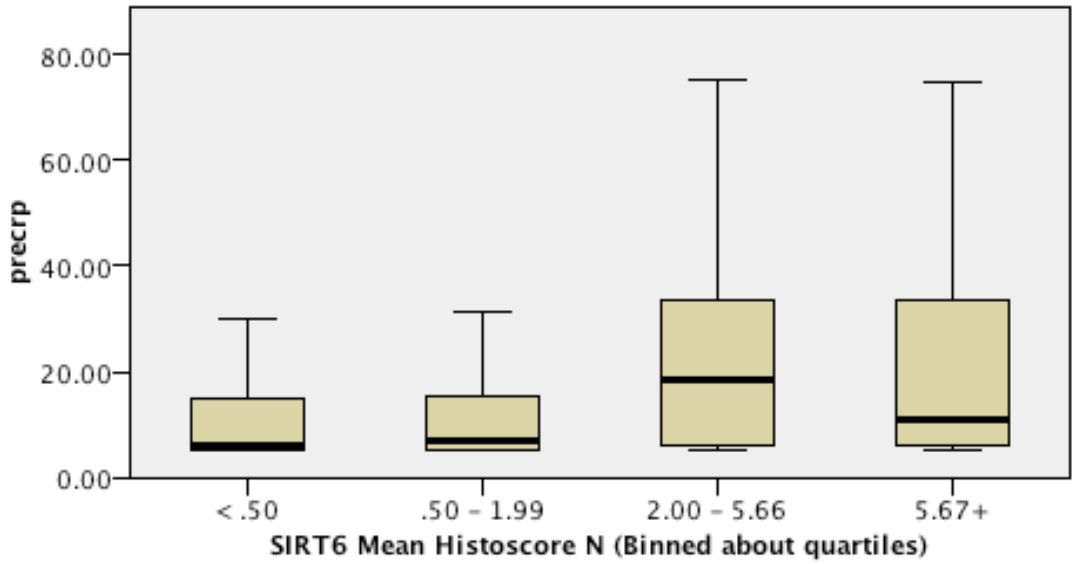

Figure 5.8 Boxplots showing distribution of CRP by quartiles of SIRT6 nuclear Histoscore. SIRT6 nuclear Histoscore above the median was associated with higher CRP (Mann-Whitney U test, p=0.00013).

Highest quartile SIRT6 cytoplasmic Histoscore was associated with low CRP (Mann-Whitney U test, p=0.00003).

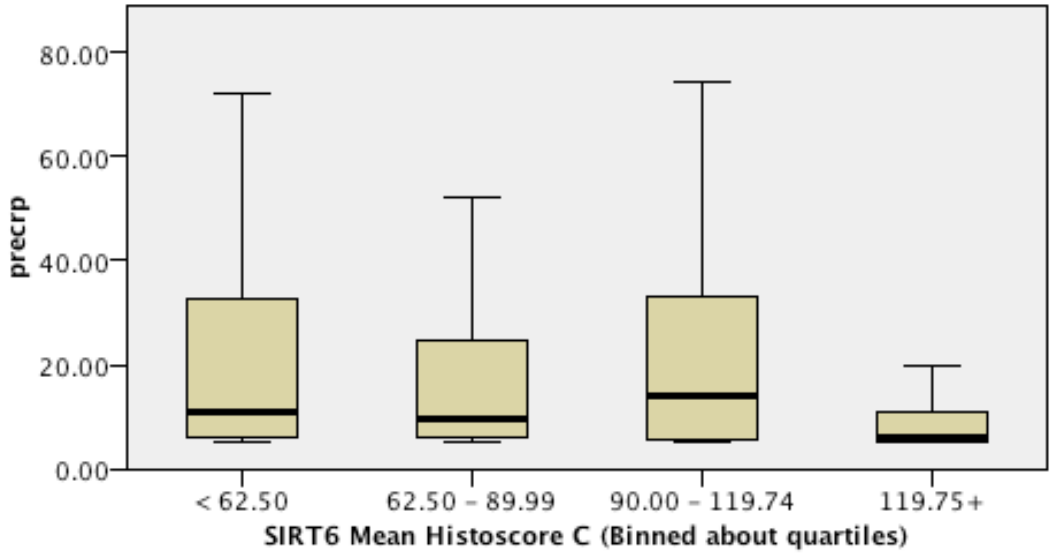

Figure 5.9 Boxplots showing distribution of CRP by quartiles of SIRT6 cytoplasmic Histoscore. Highest quartile SIRT6 cytoplasmic Histoscore was associated with lower CRP (Mann-Whitney U test, p=0.00003).

5.6.2.3 Association with serum albumin

Highest quartile SIRT6 cytoplasmic Histoscore was associated with higher serum albumin (Mann-Whitney U test, p=0.0051).

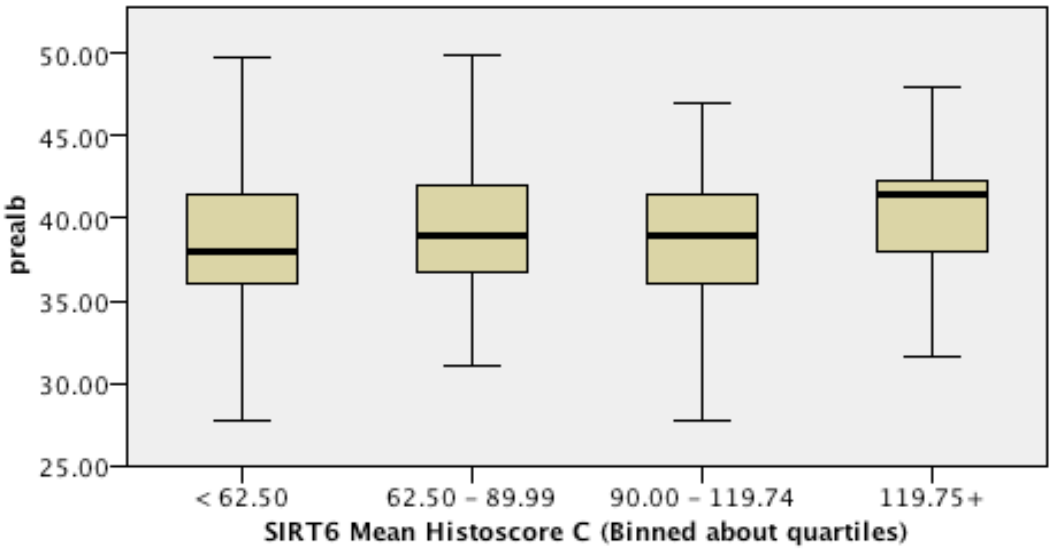

Figure 5.10 Boxplots showing distribution of preoperative albumin by quartiles of SIRT6 cytoplasmic Histoscore. Highest quartile SIRT6 cytoplasmic Histoscore was associated with higher albumin (Mann-Whitney U test, p=0.0051).

5.6.2.4 Association with modified Glasgow Prognostic Score (mGPS)

High mGPS (more inflammation) was associated with higher SIRT6 nuclear staining (Kruskal-Wallis test, p=0.0008).

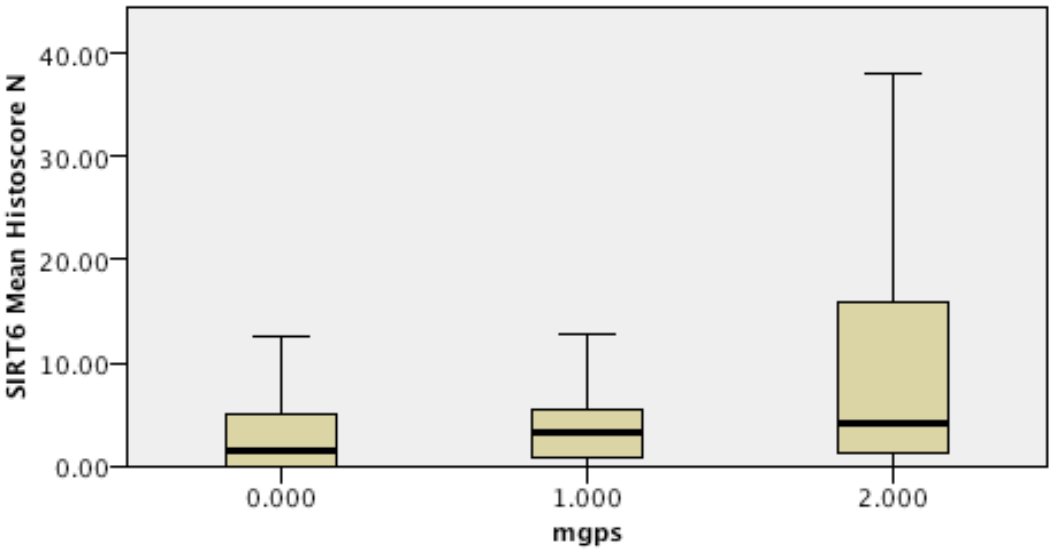

Figure 5.11 Boxplots showing distribution of SIRT6 nuclear Histoscore categorised by mGPS. Higher SIRT6 nuclear Histoscore was associated with higher mGPS score (Kruskal-Wallis test, p=0.0008).

Increasing mGPS was associated with lower SIRT6 cytoplasmic staining, (Kruskal-Wallis test, p=0.0054).

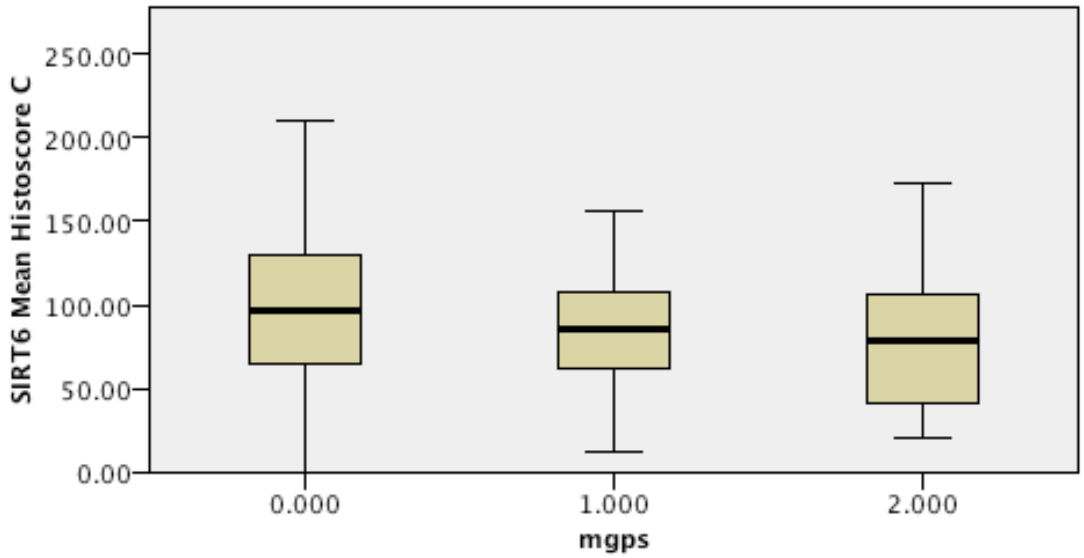

Figure 5.12 Boxplots showing distribution of SIRT6 cytoplasmic Histoscore categorised by mGPS. Lower SIRT6 cytoplasmic Histoscore was associated with higher mGPS score (Kruskal-Wallis test, p=0.0054).

5.6.3 Association with atypical inflammatory markers

5.6.3.1 Association with haemoglobin

Highest quartile SIRT6 cytoplasmic Histoscore was associated with higher haemoglobin (Mann-Whitney U test, p=0.0036).

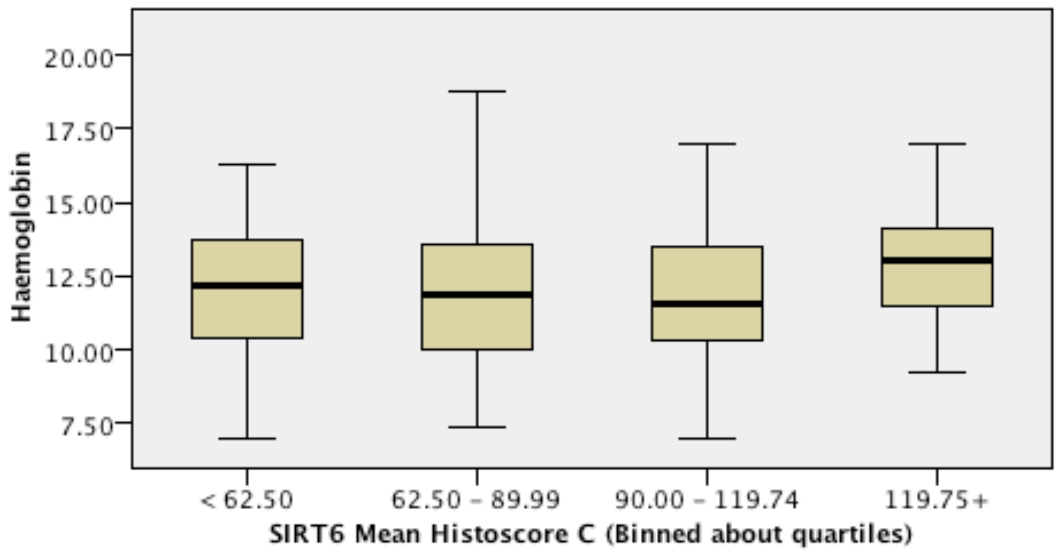

Figure 5.13 Boxplots showing distribution of preoperative haemoglobin by quartiles of SIRT6 cytoplasmic Histoscore. Highest quartile SIRT6 cytoplasmic Histoscore was associated with higher haemoglobin (Mann-Whitney U test, p=0.0036).

5.6.4 Associations with histological factors of prognostic significance in colorectal cancer

Low SIRT6 cytoplasmic staining was associated with poorly differentiated tumours (Mann-Whitney U test, p=0.0008).

Highest quartile SIRT6 nuclear staining was associated with venous invasion (Pearson Chi square, p=0.010).

		SIRT6 Nuclear Histoscore		
		3 lower quartiles	Highest quartlle	Total
Venous invasion	N	132	32	164
	Y	65	33	98
Total		197	65	262

Table 5.17 Crosstabulation of venous invasion and SIRT6 nuclear Histoscore categorised by highest quartile and lower quartiles. Highest quartile SIRT6 nuclear Histoscore was associated with venous invasion (Pearson Chi square, p=0.010).

Lowest quartile SIRT6 cytoplasmic staining is associated with high risk Petersen index (Pearson Chi square, p=0.008). This is interesting given that there is no relationship with any of the Petersen Index component measures and may reflect the incremental effects of small changes in each component. There was no similar association between high risk Petersen index and nuclear staining.

		SIRT6 Cytoplasmic Histoscore		
		Lowest quartile	Higher 3 quartiles	Total
High risk Petersen Index	N	45	166	211
	Y	20	31	51
Total		65	197	262

Table 5.18 Crosstabulation of high risk Petersen Index and SIRT6 cytoplasmic Histoscore categorised by lowest quartile and higher quartiles. Lowest quartile SIRT6 cytoplasmic Histoscore was associated with high risk Petersen Index (Pearson Chi square, p=0.008).

5.7 SIRT7 Expression in Colorectal Tumours and Relation with Clinicopathological Factors

Associations between SIRT7 nuclear and cytoplasmic Histoscores and clinicopathological factors are summarised in the following tables and statistically significant associations are subsequently discussed in more detail.

SIRT7		Nucleus	Cytoplasm
Demographic variables	Age	Pearson Chi square, p=0.204	Pearson Chi square, p=0.323
	Sex	Pearson Chi square, p=0.061	Pearson Chi square, p=0.780
	BMI	Highest quartile Histoscore associated with higher BMI. Mann-Whitney U test, p=0.012	Pearson Chi square, p=0.293
Inflammatory markers	WCC	Lowest quartile Histoscore was associated with low WCC. Mann-Whitney U test, p=0.0487	Lowest quartile Histoscore was associated with higher WCC. Mann-Whitney U test, p=0.0264
	CRP	Higher Histoscore was associated with higher CRP. Mann-Whitney U test, p=0.0224	Lower Histoscore was associated with high CRP. Mann-Whitney U test, p=0.0015
	Albumin	Pearson Chi square, p=0.996	Higher serum albumin was associated with higher quartile Histoscores. Kruskal-Wallis test, p=0.00004
	mGPS	Lowest quartile Histoscore was associated with low mGPS. Pearson Chi square, p=0.011, linear-by-linear, p=0.004	High Histoscore was associated with low mGPS. Pearson Chi square, p=0.001, linear-by-linear, p=0.00012
Atypical markers of	Hb	Pearson Chi square, p=0.111	Pearson Chi square, p=0.916
	Smoking status	Pearson Chi square, p=0.610	Pearson Chi square, p=0.254
Presentation		Pearson Chi square, p=0.522	Pearson Chi square, p=0.405
Charlson Comorbidity Index		Pearson Chi square, p=0.560	Pearson Chi square, p=0.533
Tumour location		Pearson Chi square, p=0.927	Pearson Chi square, p=0.483

Table 5.19 Associations between SIRT7 nuclear and cytoplasmic Histoscores and demographic variables, classical and atypical markers of inflammation, presentation, comorbidity and tumour location. Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

SIRT7		Nucleus	Cytoplasm
Measures of cancer staging	Dukes' stage	Higher Histoscore was associated with higher Dukes' stage. Kruskal-Wallis test, p=0.0264	Pearson Chi square, p=0.621
	T stage	Higher quartile Histoscores were associated with higher T stage. Kruskal-Wallis tesst, p=0.049	Pearson Chi square, p=0.961
	N stage	Lowest quartile Histoscore was associated with low N stage. Pearson Chi square, p=0.006, linear-by-linear, p=0.001	Pearson Chi square, p=0.189
	M stage	Pearson Chi square, p=0.329	Pearson Chi square, p=0.326
Pathological prognostic markers	Differentiation	Pearson Chi square, p=0.084	Pearson Chi square, p=0.213
	Margin involvement	Pearson Chi square, p=0.419	Pearson Chi square, p=0.706
	Peritoneal involvement	Higher Histoscore was associated with peritoneal involvement. Mann-Whitney U test, p=0.0033	Pearson Chi square, p=0.740
	Tumour perforation	Pearson Chi square, p=0.932	Pearson Chi square, p=0.480
	Venous invasion	Higher quartile Histoscores were associated with venous invasion. Mann-Whitney U test, p=0.0291	Higher Histoscores were associated with venous invasion. Mann-Whitney U test, p=0.0034
	High risk Petersen index	Lowest quartile Histoscore was associated with low risk PI. Mann-Whitney U test , p=0.0003	Pearson Chi square, p=0.347
Peritumoural Inflammation		Pearson Chi square, p=0.308	Pearson Chi square, p=0.793

Table 5.20 Associations between SIRT7 nuclear and cytoplasmic Histoscores and accepted factors of prognostic significance in colorectal cancer (cancer staging systems and histopathological markers). Pearson Chi square p-values are for sirtuin Histoscores split about the median unless otherwise indicated.

5.7.1 Association with inflammatory markers

5.7.1.1 Association with white cell count

Lowest quartile SIRT7 cytoplasmic staining was associated with higher WCC (Mann-Whitney U test, p=0.0264).

5.7.1.2 Association with serum C-reactive protein (CRP)

Higher SIRT7 nuclear Histoscores were associated with CRP above 10 (Mann-Whitney U test, p=0.0224). Lower SIRT7 cytoplasmic Histoscores were associated with high CRP (Mann-Whitney U test, p=0.0015).

5.7.1.3 Association with serum albumin

Serum albumin increased with increasing quartiles of SIRT7 cytoplasmic Histoscores (Kruskal-Wallis test, p=0.00004).

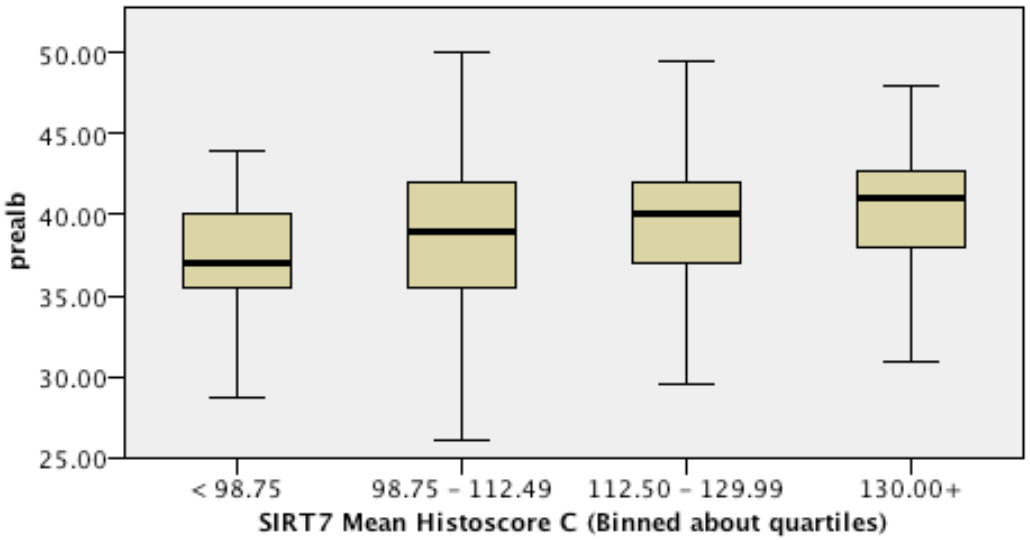

Figure 5.14 Boxplots of serum albumin categorised by SIRT7 nuclear Histoscore quartiles. Albumin increased with increasing quartiles of SIRT7 cytoplasmic Histoscores (Kruskal-Wallis test, p=0.00004).

5.7.1.4 Association with modified Glasgow Prognostic Score (mGPS)

Lowest quartile SIRT7 nuclear staining was associated with lower mGPS (Pearson Chi square, p=0.011, linear-by-linear, p=0.004).

		SIRT7 Nuclear Histoscore		
		Lowest quartile	Higher 3 quartiles	Total
mGPS	0	43	103	146
	1	14	76	90
	2	3	26	29
Total		60	205	265

Table 5.21 Crosstabulation of modified Glasgow Prognostic Score (mGPS) with SIRT7 nuclear Histoscore, categorised by lowest quartile and higher three quartiles. Lowest quartile SIRT7 nuclear staining was associated with lower mGPS (Pearson Chi square, p=0.011, linear-by-linear, p=0.004).

SIRT7 cytoplasmic staining above the median was associated with low mGPS (Pearson Chi square, p=0.001, linear-by-linear, p=0.00012).

		SIRT7 Cytoplasmic Histoscore		
		Below median	Above median	Total
mGPS	0	57	89	146
	1	52	38	90
	2	21	8	29
Total		130	135	265

Table 5.22 Crosstabulation of modified Glasgow Prognostic Score (mGPS) with SIRT7 cytoplasmic Histoscore, categorised by quartiles. Lower mGPS was associated with higher quartile SIRT7 cytoplasmic Histoscore (Pearson Chi square, p=0.001, linear-by-linear, p=0.00012.

5.7.2 Associations with accepted factors of prognostic significance in colorectal cancer

5.7.2.1 Associations with cancer stage

Lowest quartile SIRT7 nuclear Histoscore was associated with less advanced N stage (Pearson Chi square, p=0.006, linear-by-linear, p=0.001).

		SIRT7 Nuclear Histoscore		
		Lowest quartile	Higher three quartiles	Total
N stage	0	44	104	148
	1	14	76	90
	2	2	25	27
Total		60	205	265

Table 5.23 Crosstabulation of nodal (N) stage with SIRT7 nuclear Histoscore, categorised by lowest and higher three quartiles. Lowest quartile Histoscore was associated with lower N stage (Pearson Chi square, p=0.006, linear-by-linear, p=0.001).

5.7.2.2 Associations with histological factors of prognostic significance

Venous invasion was associated with heavier SIRT7 cytoplasmic staining (Mann-Whitney U test, p=0.0034).

Peritoneal involvement was associated with higher SIRT7 nuclear Histoscore (Mann-Whitney U test, p=0.0033). Higher SIRT7 nuclear Histoscore was also associated with high-risk Petersen index (Mann-Whitney U test, p=0.0003).

5.8 Discussion

5.8.1 SIRT2 tumour Histoscore and associations with inflammatory markers

Higher CRP and low albumin and the corresponding composite measure of high mGPS are markers of a systemic inflammatory response. All three were associated with extreme quartiles of SIRT2 nuclear Histoscore. Systemic inflammation has been linked with poorer outcome in colorectal cancer. The observed relationships between extreme quartiles of SIRT2 nuclear Histoscore, increased systemic inflammation and survival point to the involvement of SIRT2 in a nuclear role in this interaction.

There was no significant association between SIRT2 nuclear Histoscore and white cell count. The inflammatory response is a complex process involving many ways of responding to different threats to the organism. These parameters may measure different aspects of the inflammatory process. What determines whether SIRT2 levels in the nucleus rise rather than fall, and what downstream effects do these two outcomes have? SIRT2 deacetylates a component of NFκB and carries NFκB binding sites in its 5'UTR (Rothgiesser, Erener et al. 2010). NFκB controls many genes involved in inflammation, in cell division and in the immune response. Further investigation into the place of SIRT2 in inflammatory responses is likely to be fruitful.

5.8.2 SIRT2 tumour Histoscore and associations with accepted factors of prognostic significance in colorectal cancer

T stage provides a more detailed summary of local progression of the tumour and increasing T stage was associated with extremes of SIRT2 nuclear Histoscore. There was no significant relationship with overall Dukes' stage or with nodal involvement, which might have been expected to have more bearing on survival and thus a stronger relationship with SIRT2.

Two cellular measures of poor prognosis were associated with SIRT2 nuclear Histoscore and these may indicate useful lines of investigation to understand instead how the survival effect is manifest and how this may be modulated with therapeutic intent. Associations with poorly differentiated tumours could be linked to SIRT2 influences on HOXA10. HOXA10 is a DNA-binding transcription factor and one of the homeobox genes that govern morphogenesis in embryological development and are necessary in adult life for functional differentiation(Daftary and Taylor 2006). Aberrant regulation of such a gene could potentially contribute to the poorly differentiated phenotype of some cancer cells.

Venous invasion requires that a malignant cell has altered interaction with neighbouring cells and increased mobility, part of the epidermal-to-mesenchymal transition that underlies metastatic potential. SIRT2 is phosphorylated by cyclin-dependent kinases with measured reduction in adherence to the substratum and increased cell mobility(Pandithage, Lilischkis et al. 2008). There is associated reduction in acetylated α-tubulin. SIRT2 interaction with tubulin is likely largely the preserve of the cytoplasmic fraction, but it could relate to SIRT2 nuclear Histoscores, if subcellular distribution of SIRT2 is conjointly controlled.

SIRT2 has several other targets in the nucleus, which in turn have widespread effects on translation of genes from pathways governing a range of processes including apoptosis and cell division. H3K56Ac levels are increased in many types of cancer(Vempati, Jayani et al. 2010) and the reduced levels of SIRT2 in colorectal cancer would facilitate this, by reducing the deacetylation of this substrate. H3K56Ac localises to areas of DNA damage along with p53, also subject to regulation by deacetylation by SIRT2(Jin, Kim et al. 2008). The details of the effects of the interactions between H3K56Ac and p53 are not known but

they certainly have the capacity to significantly influence gene transcription to produce the widespread changes involved in acquisition of metastatic potential.

Taken together these results support a detrimental effect of extreme SIRT2 nuclear Histoscores on survival. Highest quartile SIRT2 nuclear Histoscore may be the worst of the two abnormalities as it is also associated with markers of inflammation and of histopathologically defined poor prognosis.

It must be noted that these associations do not imply causality. Further investigation is needed to clarify the mechanisms by which SIRT2 nuclear expression impacts on survival from colorectal cancer. The recently published interaction between SIRT2 and K-RAS is an exciting extension to this(Yang, Laurent et al. 2013). SIRT2 deacetylates K-RAS which has been known for some time to affect survival from colorectal cancer, and deacetylation enhances K-RAS transforming activity.

5.8.3 SIRT3 tumour Histoscore and associations with demographic variables

5.8.3.1 Age

Previous reports that SIRT3 is the only sirtuin associated with age in humans(Rose, Dato et al. 2003; Bellizzi, Rose et al. 2005) were not borne out by this dataset, as the trend to older age with higher cytoplasmic expression of SIRT3 did not reach statistical significance. Interestingly, the work by Rose et al noted the association between SIRT3 polymorphisms and age in males only. The cohort examined here was equally mixed in terms of sex. There was no association with nuclear SIRT3. It is possible that the association with chronological age might be mediated through SIRT3 functions in the mitochondria, by modulating redox homeostasis. The protective effect of oestrogen on redox homeostasis may reduce the magnitude of this interaction in females.

5.8.4 SIRT3 tumour Histoscore and associations with inflammatory markers

Higher SIRT3 cytoplasmic Histoscores were associated with lower white cell count and CRP. The inverse relationship between SIRT3 cytoplasmic Histoscores and white cell count may relate to the nuclear targeting of SIRT3 in some conditions, such as inflammation when there may be a link with NFκB signalling and increased oxidative stress. The relationship between SIRT3 nuclear Histoscores and white cell count did not reach statistical significance. This may relate to the relatively low overall nuclear staining for SIRT3 in that the Histoscore measurement may not have been precise enough to detect relevant staining, or to differentiate at this level.

Higher haemoglobin associates with higher SIRT3 cytoplasmic expression and overall, higher SIRT3 cytoplasmic expression is associated with more favourable physiological parameters.

5.8.5 SIRT3 tumour Histoscore and associations with accepted factors of prognostic significance in colorectal cancer

Lower cytoplasmic staining for SIRT3 was associated with poorly differentiated tumours, while higher nuclear staining was associated with venous invasion. In keeping with the associations with inflammatory markers, lower cytoplasmic staining (and in this context, higher nuclear staining) seem to be correlated with less favourable pathological features.

Of note, this model does not accord with the association between lower nuclear protein expression and worse survival. The significance of the associations with serum and pathological markers for the patient may be questioned, but it must be remembered that the association between low nuclear SIRT3 staining and worse survival was not maintained on multivariate analysis, whereas the association between survival and systemic inflammation (CRP) was robust.

Notwithstanding, lower SIRT3 expression in the cytoplasm is in keeping with preferential glycolytic metabolism in cancer cells. SIRT3 tends to shift the balance towards other substrates(Finley, Carracedo et al. 2011), a useful trait in times of glucose scarcity but one that may be suppressed in cancer cells. The shift towards glycolysis may be a prerequisite for increased cell division and local invasion.

Nuclear targets of SIRT3 include histone 3 and 4: increased deacetylation of these histones will modulate expression of a range of genes. Specific targets include a sulphotransferase (GAL3ST1) known to be upregulated in renal cell carcinoma, although there is little information on its downstream effects(Iwahara, Bonasio et al. 2012). Another target is focal adhesion kinase, central to cell adhesion and spreading processes including fast turnover of focal adhesions. Depletion of this protein in breast cancer cells reduced invasive cell migration(Chan, Cortesio et al. 2009) and it is possible that increased expression as a result of SIRT3 activity in the nucleus could be instrumental in aiding local invasion and underlie the association with increased T stage and possibly the link with venous invasion.

A mechanistic link between poor differentiation and low cytoplasmic staining is less easy to see. It is difficult to define poor differentiation in molecular biological terms but this may change as more molecular markers come into regular use.

5.8.6 SIRT4 tumour Histoscore and associations with inflammatory markers

Cytoplasmic localisation of SIRT4 in colorectal tumour cells appears to accompany a reduced systemic inflammatory response. Systemic inflammation is associated with worse outcome in colorectal cancer but there are no data at present to link SIRT4 mechanistically to this effect. This was the only association identified with SIRT4. Data in the following chapter on sirtuin interrelationships might indicate that SIRT4 has a regulatory effect on other sirtuins, rather than any demonstrable impression on other cellular pathways investigated here.

SIRT4 decreases mitochondrial capacity to store calcium: higher levels of SIRT4 in the cytoplasm may aid cancer cell survival when calcium regulation is perturbed. This is normally a late stage in cancer however, and was not a common feature in the cohort of patients with operable tumours under investigation. Further research on the functions and interactions of SIRT4 may enable more useful interpretation of its role in systemic inflammation in colorectal cancer.

5.8.7 SIRT5 tumour Histoscore and associations with inflammatory markers

Lowest quartile SIRT5 cytoplasmic staining was associated with higher white cell count and CRP, both markers of a systemic inflammatory response, carrying a poorer prognosis. SIRT5 activity is important in regulating the use of alternative sources of energy, while mitigating the build-up of toxic by-products such as ammonia. The general reduction of SIRT5 expression in the cytoplasm may limit the tumour cell's ability to deal with periods of nutrient restriction, or high usage in inflammation.

This could incline to reliance on the simpler process of glycolysis as is seen in tumour cells. The Warburg effect describes the dependence on glycolysis even in the presence of preferable substrates(Warburg 1956), but that dependence is equally useful in the absence of such substrates. Tumour cells are uncoupled from the regulation essential to mediate the interdependence of cells forming part of a whole body, and thus may not avail of the signalling systems which mobilise stored nutrients to provide for excess requirements. Escape from this regulation is one of the characteristics of malignantly transformed cells(Hanahan and Weinberg 2011).

5.8.8 SIRT6 tumour Histoscore and associations with demographic variables

5.8.8.1 Age, sex, BMI

Although SIRT6 levels decline in human fibroblasts in line with the development of replicative senescence (Mao, Tian et al. 2012) there was no association between staining in colorectal tumour cells and chronological age.

Higher quartile SIRT6 nuclear staining associates with higher BMI. SIRT6 and SIRT1 have a role in lipid metabolism and it may be that background higher levels of SIRT6 allow organisms to grow larger, through efficient use of lipids. It may be more simply that such organisms store more lipid. Given that the interaction with SIRT1 is central to these lipid regulatory networks, it is a pity that no SIRT1 staining could be completed for this cohort.

5.8.9 SIRT6 tumour Histoscore and associations with inflammatory markers

Higher nuclear SIRT6 Histoscore was associated with lower CRP and lower mGPS. Higher cytoplasmic SIRT6 Histoscore was associated with lower white cell count, CRP below 10, higher albumin and lower mGPS. Higher cytoplasmic SIRT6 Histoscore also associated with higher haemoglobin. Thus it appears that markers of increased systemic inflammation were reflected in lower nuclear SIRT6 staining and lower cytoplasmic staining.

SIRT6 is known to have an inhibitory effect on expression of NFκB genes and TNFα, in endothelial cells and cells of the amnion(Lappas 2012; Lim, Barker et al. 2013). In idiopathic pulmonary fibrosis, diseased lung tissue increases expression of SIRT6, which can inhibit senescence and influence production of secreted cytokines(Minagawa, Araya et al. 2011). Higher SIRT6 in colorectal cancer may be acting in a similar way, dampening an inflammatory response.

Either way, the effect of SIRT6 cytoplasmic and nuclear protein levels may reflect changes with prognostic effects, as systemic inflammatory response is associated with poorer tumour outcomes. The relationship between haemoglobin and cytoplasmic SIRT6 Histoscore may indicate an alteration in tissue function at a more constitutive level. More data on the significance of SIRT6 protein in the cytoplasm is necessary before further extrapolation.

5.8.10 SIRT6 tumour Histoscore and associations with accepted factors of prognostic significance in colorectal cancer

Highest quartile nuclear SIRT6 Histoscores were associated with venous invasion. Lower cytoplasmic SIRT6 staining was associated with poorly differentiated tumours. Although there is no statistically significant effect on survival, it appears that higher nuclear SIRT6 staining is associated with a range of poor prognostic indicators, at both local and systemic levels. Conversely, higher cytoplasmic staining is associated with factors usually linked to better outcomes at systemic level but with local histopathological markers of poor prognosis. Immunohistochemistry analysis in a very similar cohort of 253 colorectal cancer cases reported reduced staining for SIRT6 overall, but improved outcome for

node-positive patients with higher SIRT6 levels (Sebastian, Zwaans et al. 2012). The localisation of the staining was not further characterised so it is difficult to say whether these results correspond to the data presented here.

SIRT6 has several known nuclear functions in maintenance of DNA and telomere length. The specific role played by SIRT6 in maintenance of telomere length is not understood but involves stabilisation and prevention of replicative senescence which must be a priority for tumour cells. In rapidly dividing cells, there is likely to be an increased requirement for DNA repair and increased SIRT6 staining in the nucleus could reflect more mobilisation to compensate for this. Homologous recombination, the more precise form of DNA repair decreases with onset of replicative senescence. SIRT6 thus has a twofold effect on DNA integrity, since it is central to the recruitment of the DSB repair machinery. SIRT6 also has a key although undefined part to play in base excision repair.

Although none of this information provides a direct link to the association between venous invasion and SIRT6 nuclear expression, increased tumour cell survival and division is necessary to facilitate tumour spread. Research into the dynamics of SIRT6 movement at a subcellular level and to clarify its roles in the nucleus are likely to prove fruitful in determining how to influence its regulatory actions within tumour cells.

5.8.11 SIRT7 tumour Histoscore and associations with demographic variables

5.8.11.1 Age

SIRT7 staining in colorectal epithelium appears to be similar in distribution to that reported in fibroblasts (Kiran, Chatterjee et al. 2013). However, the reduced nucleolar staining associated with replicative senescence in fibroblasts is not reflected in substantially reduced nuclear Histoscores in colorectal tumour tissue. There was no association between nuclear or cytoplasmic SIRT7 Histoscores and age in this cohort of tissue samples. Although cancer cells are thought to represent aged tissue, this may not simply be ageing through repeated cell divisions. Of note, premature ageing does not produce the same reduction in nucleolar staining as replicative senescence, and it may be a better model of the aged nature of cancer cells.

5.8.12 SIRT7 tumour Histoscore and associations with inflammatory markers

Lower SIRT7 nuclear Histoscores were associated with lower mGPS. Lower SIRT7 cytoplasmic Histoscores were associated with high CRP, low albumin and higher mGPS. Taken together these results suggest a reciprocal relationship between SIRT7 subcellular localisation and systemic inflammatory status. Thus high nuclear SIRT7 Histoscore is associated with higher systemic inflammation and by inference, poorer prognosis, given reported relationships between outcome and systemic inflammatory response in colorectal cancer (Roxburgh, Salmond et al. 2009).

The reciprocal variation in nuclear and cytoplasmic SIRT7 Histoscores is reminiscent of the reciprocal change in nuclear and cytoplasmic forms of SIRT7 reported by Kiran et al in fibroblasts, in association with replicative senescence(Kiran, Chatterjee et al. 2013). The change seen in these colorectal cancer specimens is in the opposite direction, with increasing SIRT7 nuclear Histoscore associated with increasing systemic inflammatory features. However, the motif worth further investigation is of 2 pools of SIRT7 whose cellular localisation changes in association with cellular responses. Whether this change in localisation is causative or reactive is not clear. Indeed, it is not known whether the cytoplasmic fraction of SIRT7 is an inert sequestered pool or an enzymatically active agent regulating as yet unidentified processes. The former might be possible given the lack of antibody reactivity of cytoplasmic SIRT7 in epithelial cells, although this fraction was detectable on immunoblotting.

5.8.13 SIRT7 tumour Histoscore and associations with accepted factors of prognostic significance in colorectal cancer

Increasing SIRT7 nuclear Histoscores were associated with increasing N stage, peritoneal invasion and high-risk Petersen Index. There was also an association between increased SIRT7 cytoplasmic Histoscore and venous invasion. Given the several associations between histopathological features of worse prognosis and SIRT7 nuclear staining, it is surprising that there is not a survival disadvantage for patients with higher SIRT7 nuclear Histoscores. However, as indicated by Barber et al, it is possible that SIRT7 also has a role in maintaining the

transformed phenotype through deacetylation of acetylated lysine 18 of histone H3 at a specific set of gene targets (Barber, Michishita-Kioi et al. 2012). They did not find any evidence of a role for SIRT7 in initiating transformation of cells.

Tumour cells have an active TCA cycle and appear to depend heavily on glutamine metabolism for production of NADH. Although there are significant biosynthetic requirements for expansion of cell numbers, the rate of these reactions exceeds that requirement. Handling this increased throughput of substrate and the increased synthetic requirements of malignant transformation must require some increase in ribosomal protein synthesis. This may explain at least part of the increased nuclear staining for SIRT7.

The genes targeted by SIRT7 comprise a set of ribosomal proteins whose expression is repressed by its action. While this is unexpected, there are also reports of links between ribosomal proteins and cancer progression possibly through imbalances in regulation of translation.

Further research into the function of the SIRT7 nuclear and cytoplasmic fractions, and the mobilisation or exchange between these two pools in response to cellular conditions is likely to prove and exciting and fruitful area of research.

6 Sirtuin Interrelationships

6.1 Integrated control of protein interactions

Proteins that act in same pathways may be expected to have correlated expression levels (Clarke, Ressom et al. 2008). However, reverse causation does not mean that proteins that are correlated will interact. Bearing in mind also that proteins that are correlated may act in the same pathways, but do not necessarily interact directly with each other, correlations between protein levels may be a useful starting point to look for pathways of interaction.

Some documented interactions have already been reported between sirtuins; these are summarised here.

6.1.1 Direct regulation within the sirtuin family

There is evidence of direct negative regulation of SIRT1 and SIRT3 by SIRT4 in mouse hepatocytes (Chen, Fang et al. 2010). SIRT1 has positive regulatory effect on SIRT6 in TLR4 activation in a human model of inflammation (using THP1 human promonocytes) (Liu, Vachharajani et al. 2012). SIRT1 also has positive regulatory effect on SIRT6 via complex formation with FOXO3a and NRF1(Kim, Xiao et al. 2010).

Coexpression of SIRT3 and SIRT5 results in translocation of SIRT3 to the nucleus in mice (Nakamura, Ogura et al. 2008). While this interaction does not impact on SIRT3 expression levels, it may regulate function within the cell by modulating subcellular localisation.

6.1.2 Common substrates within the sirtuin family

There are several reports of sirtuins that have opposing effects on one substrate, glutamate dehydrogenase (GDH) was first identified as a target for inhibition by SIRT4 (Haigis, Mostoslavsky et al. 2006). SIRT3 has since been shown to activate GDH(Lombard, Alt et al. 2007). SIRT1 and SIRT7 interact with RNAPol1 to inhibit and activate it respectively (Muth, Nadaud et al. 2001; Ford, Voit et al. 2006).

There are several lines of evidence that more than one sirtuin targets the same substrate with similar effect. This may reflect a degree of redundancy in crucial pathways. Different subcellular localisations of sirtuins may also require this arrangement to regulate separate pools of substrate, and this has been suggested as the underlying evolutionary rationale for the existence of a number of similar or identical substrates for SIRT1 and SIRT3. For example, SIRT1 and SIRT3 both deacetylate acetyl CoA synthase 1 (AceCS1) in vitro (Hirschey, Shimazu et al. 2011). In vivo SIRT3 appears not to show this activity, possibly because of its largely mitochondrial location; instead, SIRT3 deacetylates AceCS2 in mitochondria. These authors also reported on HMGCS as a target for both SIRT1 and SIRT3. Other substrates common to both SIRT1 and SIRT3 include FOXO3a, chaperonin, heat shock protein 70 (Hsp70) and uridine diphosphoglucose dehydrogenase (Jacobs, Pennington et al. 2008; Law, Liu et al. 2009).

SIRT1, SIRT6, SIRT2 all deacetylate H3K56 (Das, Lucia et al. 2009; Michishita, McCord et al. 2009; Vempati, Jayani et al. 2010). PGC1α is an important transcription regulator and a common target of SIRT1 and SIRT6. Interestingly however, it is directly deacetylated by SIRT1 to produce activation, but the opposite negative regulatory effect is achieved by SIRT6 deacetylation of GCN5, which acetylates PGC1α(Rodgers and Puigserver 2007; Dominy, Lee et al. 2012).

The specific interaction of a sirtuin with a particular substrate may not be limited to deacetylation with the discovery that SIRT5, a weak deacetylase in general and in comparison to other sirtuins, is an efficient desuccinylase and demalonylase(Park, Chen et al. 2013). This widens the scope of multiple interactions with a single target.

6.1.3 Common regulators of sirtuins

The ubiquitous control of sirtuin activity by ambient NAD levels must be taken into account. Reports of common regulators of multiple sirtuins are more sparse. SIRT2 and SIRT7 are both phosphorylated by the CDK1-cyclin B pathway (Grob, Roussel et al. 2009).

6.1.4 Current evidence of sirtuin interrelationships

Previous research has shown evidence of strong and significant correlations between sirtuins at the gene transcription level, i.e. between levels of sirtuin mRNA (Maxwell 2013).

Posttranscriptional processing adds a level of precise regulation after transcription, and subcellular localisation of proteins, yet another layer. It seemed reasonable to investigate whether the correlations reported at mRNA level persisted to protein level, and how they may be manifest through the filter of subcellular localisation.

6.2 Analysis of sirtuin interrelationships in RNA and protein

Histoscore data did show significant correlations between protein expression of several sirtuins in both normal and tumour tissue. Correlations between sirtuin mRNA expression previously published (Maxwell 2013) were represented as a network. Networks were constructed for normal and tumour mRNA. Similar networks were constructed using Histoscores for each combination of sirtuin and subcellular localisation to build a picture of sirtuin protein expression. At this level, a second dimension – that of subcellular localisation - was incorporated, such that SIRT2C represents SIRT2 expression in the cytoplasm, and SIRT2N represents SIRT2 expression in the nucleus.

A comparison was performed of sirtuin interrelationships in RNA and protein expression in normal tissue, and of sirtuin interrelationships in RNA and protein expression in tumour tissue, using these networks. Finally, sirtuin protein interrelationships in normal and tumour tissue were compared.

mRNA data was available for all 7 sirtuins, and protein data for sirtuins 2-7. Thus multiple correlations were required to construct each network. The statistical analysis used to interpret the results is discussed in the Methods.

6.3 Comparison of Sirtuin RNA Interrelationships and Sirtuin Protein Interrelationships in Normal Colorectal Tissue

Strong and statistically significant correlations between mRNA expression of different sirtuins in normal colorectal tissue were represented as a network. Strong and statistically significant correlations between protein expression of different sirtuins in normal colorectal tissue were represented similarly.

Note that there was no data for SIRT1 protein expression. Some correlations seem to be maintained from mRNA to protein expression, but in some cases there was no evidence of correlation at the protein level. There were also some correlations at protein level, not seen between sirtuin mRNA levels. Protein expression may give a better indication of the functional effect of the sirtuins within the cell. Differential protein expression levels in colorectal cancer compared to normal colorectal tissue suggest altered posttranslational processing in colorectal cancer cells. Posttranslational processing alterations in colorectal cancer cells may be a useful avenue for therapeutic research involving sirtuins, if the altered protein levels proved to be of functional relevance.

The correlations between sirtuins at mRNA and protein levels are tabulated for easier recognition of conserved and differing associations.

	Correlation		Correlation
mRNA	SIRT2 and SIRT3	Protein	.
	SIRT2 and SIRT4		.
	SIRT3 and SIRT4		.
	SIRT3 and SIRT5		SIRT3N and SIRT5C
	SIRT3 and SIRT7		SIRT3C and SIRT7C
	SIRT5 and SIRT7		.
	SIRT6 and SIRT7		.
	.	Novel protein-only correlations	SIRT2N with SIRT7C
	.		SIRT4N with SIRT4C

Table 6.1 Comparison of correlations observed at mRNA and protein level between different sirtuins in normal colorectal tissue, listing the correlations represented in the networks above. The correlations seen at mRNA level but not at protein level or vice versa are marked with a dot.

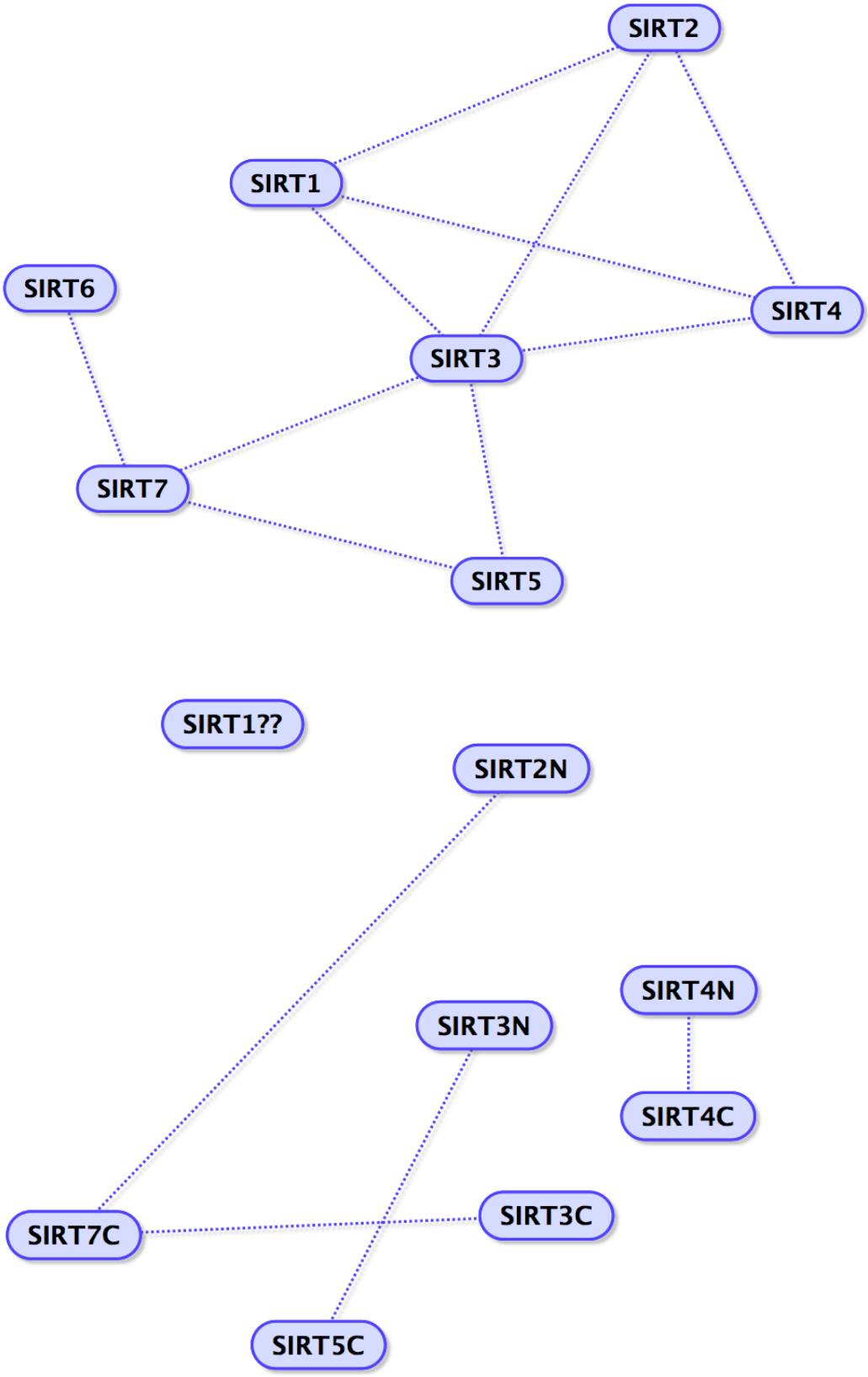

Figure 6.1 Two networks showing strong and significant correlations between mRNA levels (upper) and protein levels (lower) for the sirtuins in normal colorectal tissue. The N and C suffixes in the protein designators indicate whether the Histoscores refer to nuclear or cytoplasmic expression.

6.4 Comparison of Sirtuin RNA Interrelationships and Sirtuin Protein Interrelationships in Colorectal Tumour Tissue

The correlations between sirtuins at mRNA and protein levels are tabulated for easier recognition of the few conserved and differing associations. Correlations at the protein level with moderate/strong correlation coefficients are highlighted.

Strong and statistically significant correlations between mRNA expression of different sirtuins in colorectal tumour tissue were represented as a network. Strong and statistically significant correlations between protein expression of different sirtuins in colorectal tumour tissue were represented similarly.

In comparison with the mRNA network in normal tissue there appears to be a gain in complexity of the mRNA network in tumour tissue. However, this decidedly does not translate into a more complex network of correlations at the protein level.

	Correlation		Correlation
RNA	SIRT2 and SIRT3	Protein	SIRT2C and SIRT3N
			SIRT2C and SIRT3C
	SIRT2 and SIRT4		SIRT2N and SIRT4N
			SIRT2C and SIRT4C
	SIRT2 and SIRT5		SIRT2C and SIRT5C
	SIRT2 and SIRT6		SIRT2C and SIRT6C
	SIRT2 and SIRT7		SIRT2N and SIRT7N
			SIRT2C and SIRT7C
	SIRT3 and SIRT4		SIRT3N and SIRT4N
			SIRT3N and SIRT4C
			SIRT3C and SIRT4C
	SIRT3 and SIRT5		SIRT3N and SIRT5N
	SIRT3 and SIRT6		SIRT3N and SIRT6N
			SIRT3N and SIRT6C
			SIRT3C and SIRT6C
	SIRT3 and SIRT7		SIRT3N and SIRT7N
			SIRT3C and SIRT7C
	SIRT4 and SIRT6		SIRT4N and SIRT6N
			SIRT4C and SIRT6C
	SIRT5 and SIRT6		SIRT5N and SIRT6N
			SIRT5C and SIRT6C
	SIRT5 and SIRT7		SIRT5C and SIRT7C
	SIRT6 and SIRT7		SIRT6N and SIRT7N
			SIRT6C and SIRT7N
			SIRT6C and SIRT7C
	.	Novel protein-only correlations	SIRT2N with SIRT2C
	.		SIRT3N with SIRT3C
	.		SIRT6N with SIRT6C
	.		SIRT7N with SIRT7C
	.		SIRT4C and SIRT5C
			SIRT4C and SIRT7N
			SIRT4C and SIRT7C

Table 6.2 Comparison of correlations observed at mRNA and protein level between different sirtuins in colorectal tumour tissue. The correlations seen at mRNA level but not at protein level or vice versa are marked with a dot. Statistically significant correlations between sirtuin protein levels with correlation coefficients <0.35 are suppressed.

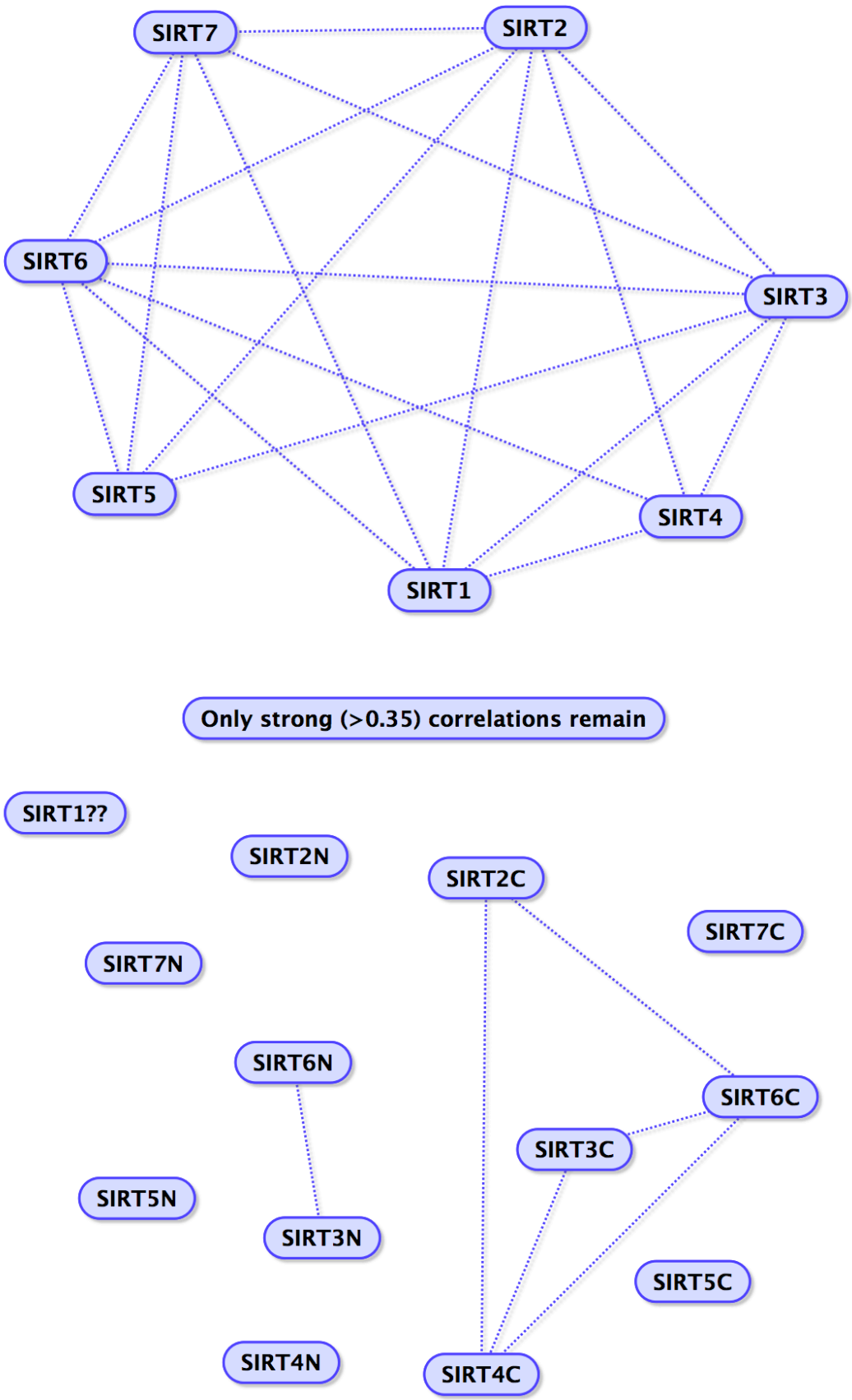

Figure 6.2 Two networks showing strong and significant correlations between mRNA levels (upper) and protein levels (lower) for the sirtuins in colorectal tumour tissue. The N and C suffixes in the protein designators indicate whether the Histoscores refer to nuclear or cytoplasmic expression. There were many fewer strong correlations in the protein network.

6.5 Comparison of Sirtuin Protein Interrelationships in Normal Colorectal Tissue and Colorectal Tumour Tissue

The significant correlations, correlation coefficients and p-values for normal and tumour tissue are tabulated with strong correlations highlighted. Strong and statistically significant correlations between protein expression of different sirtuins in colorectal normal and tumour tissue were represented as networks as in previous sections, and were compared. It is important to note that there is no data for SIRT1 protein expression.

On comparison with the sirtuin network in normal tissue, it is worth noting that there is no commonality in strong correlations between normal and tumour tissue. In other words, all the sirtuin interrelationships seen in normal tissue are lost in tumour tissue, and some new interrelationships are gained.

The functional significance of these changes, the molecular interactions underlying and explaining them and the position of SIRT1 in the network are necessary for accurate interpretation of these results. Although weaker correlations are generally not felt to have functional relevance, the presence of so many more marginal relationships between proteins in colorectal tumour cells (not represented in these networks) may provide a useful picture of the breakdown of clear and precise signalling into noise in tumour cells.

Normal tissue				Tumour tissue			
Correlations		Rho	p value	Correlations		Rho	p value
SIRT2N				SIRT2N	SIRT2C	.253**	0.00002813
SIRT2N				SIRT2N	SIRT4N	.241**	0.00007120
SIRT2N				SIRT2N	SIRT7N	.234**	0.00012232
SIRT2N	**SIRT7C**	**.683****	**0.00065208**	SIRT2N			
SIRT2C				SIRT2C	SIRT3N	-.206**	0.00072281
SIRT2C				SIRT2C	SIRT3C	.339**	0.00000001
SIRT2C				**SIRT2C**	**SIRT4C**	**.470****	**0.00000000**
SIRT2C				SIRT2C	SIRT5C	.181**	0.00317293
SIRT2C				**SIRT2C**	**SIRT6C**	**.351****	**0.00000001**
SIRT2C				SIRT2C	SIRT7C	.205**	0.00079613
SIRT3N				SIRT3N	SIRT3C	-.235**	0.00010933
SIRT3N				SIRT3N	SIRT4N	.256**	0.00002332
SIRT3N				SIRT3N	SIRT4C	-.248**	0.00004163
SIRT3N				SIRT3N	SIRT5N	.210**	0.00058052
SIRT3N	**SIRT5C**	**.563****	**0.00974608**	SIRT3N			
SIRT3N				**SIRT3N**	**SIRT6N**	**.466****	**0.00000000**
SIRT3N				SIRT3N	SIRT6C	-.301**	0.00000069
SIRT3N				SIRT3N	SIRT7N	.265**	0.00001231
SIRT3C				**SIRT3C**	**SIRT4C**	**.563****	**0.00000000**
SIRT3C				**SIRT3C**	**SIRT6C**	**.460****	**0.00000000**
SIRT3C	**SIRT7C**	**.581****	**0.00461773**	SIRT3C	SIRT7C	.321**	0.00000009
SIRT4N	**SIRT4C**	**.576****	**0.00501604**	SIRT4N			
SIRT4N				SIRT4N	SIRT6N	.279**	0.00000439
SIRT4C				SIRT4C	SIRT5C	.274**	0.00000535
SIRT4C				**SIRT4C**	**SIRT6C**	**.438****	**0.00000000**
SIRT4C				SIRT4C	SIRT7N	-.160**	0.00886534
SIRT4C				SIRT4C	SIRT7C	.284**	0.00000258
SIRT5N				SIRT5N	SIRT6N	.231**	0.00015750
SIRT5C				SIRT5C	SIRT6C	.189**	0.00204316
SIRT5C				SIRT5C	SIRT7C	.218**	0.00036046
SIRT6N				SIRT6N	SIRT6C	-.274**	0.00000657
SIRT6N				SIRT6N	SIRT7N	.305**	0.00000054
SIRT6C				SIRT6C	SIRT7N	-.220**	0.00034230
SIRT6C				SIRT6C	SIRT7C	.335**	0.00000003
SIRT7N				SIRT7N	SIRT7C	.197**	0.00125036

Table 6.3 Statistically significant correlations (p<0.01), Spearman correlation coefficients (rho) and p-values for sirtuin Histoscores in the nucleus and cytoplasm for normal and tumour tissue. The N and C suffixes in the protein designators indicate whether the Histoscores refer to nuclear or cytoplasmic expression. Strong correlations are highlighted.

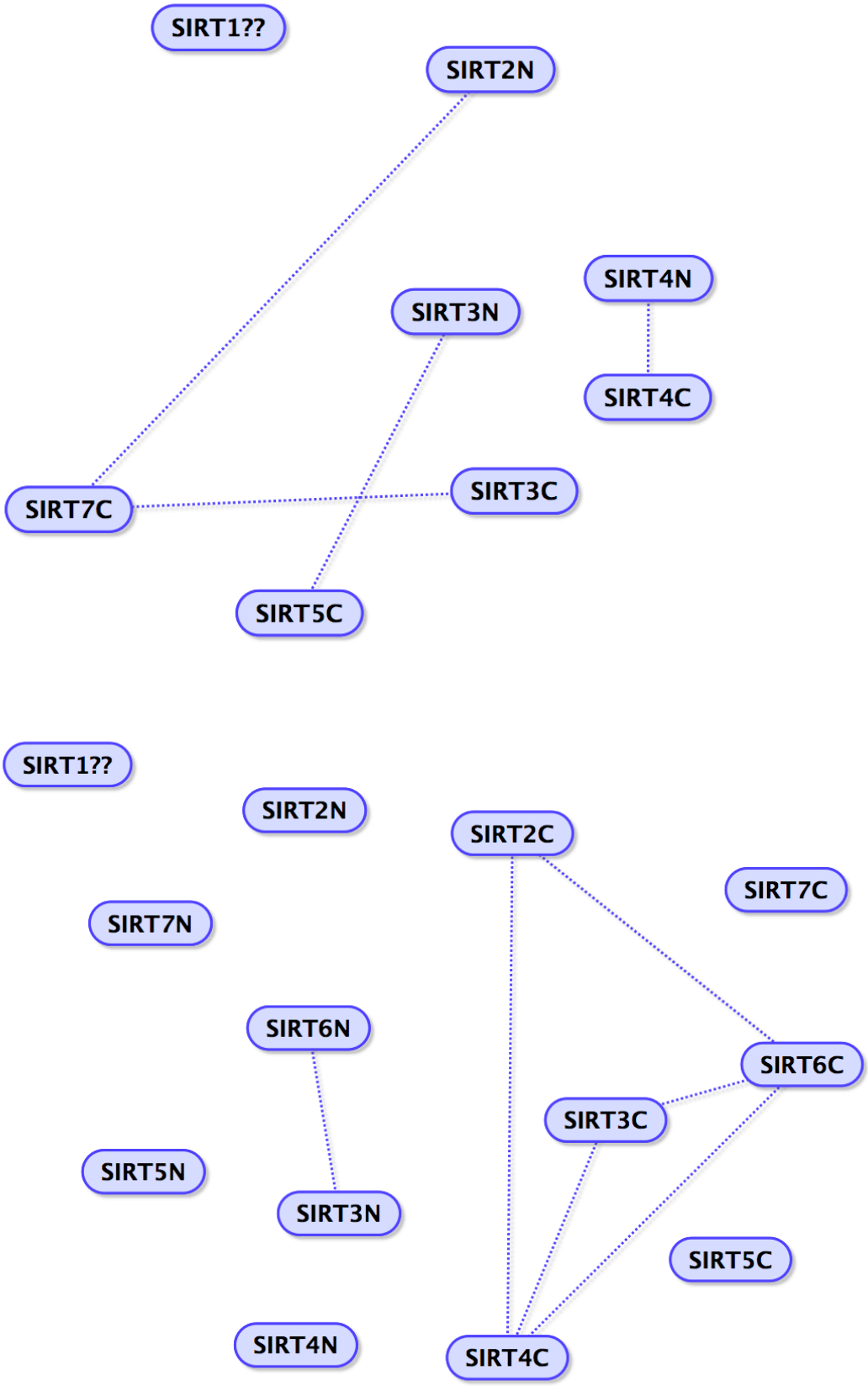

Figure 6.3 Strong and significant correlations between sirtuin Histoscores in normal colorectal tissue (upper) and colorectal tumour tissue (lower) are represented as sirtuin protein networks. The N and C suffixes in the protein designators indicate whether the Histoscores refer to nuclear or cytoplasmic expression.

6.6 Discussion

6.6.1 Functional significance of mRNA and protein networks

The correlations between level of expression of different sirtuins identified here cannot be taken as proof of any real mechanism of interaction but they do indicate a direction for further research. Correlations which persist at both mRNA and protein level are most likely to reflect a relationship of functional significance, and a particularly fruitful subject for investigation.

Evidence in support of some interactions between sirtuins has been summarised earlier. Several of these interactions involve SIRT1, and the lack of protein data for SIRT1 limits the degree to which that evidence can be applied to these results. However, in terms of correlations between sirtuin mRNA levels, this is not a factor.

6.6.1.1 Direct interactions between sirtuins

There is a correlation between mRNA levels of SIRT4 and SIRT1, and between SIRT4 and SIRT3, in line with the reports of regulation of SIRT1 and SIRT3 levels by SIRT4 in mouse hepatocytes(Chen, Fang et al. 2010). There is however no correlation to match with the relationship between SIRT1 and SIRT6 reported in human promonocytes in response to TLR4 activation (Liu, Vachharajani et al. 2012). This may be explained by a lack of inflammation, or (given the baseline level of inflammation associated with cancer) to a tissue-specific response. The relationship between SIRT1 and SIRT6 reported in human promonocytes may not be seen in colorectal epithelial cells.

There is a correlation between SIRT3 and SIRT5 mRNA levels. This may be relevant to reports that coexpression of these two sirtuins results in localisation of SIRT3 in the nucleus(Nakamura, Ogura et al. 2008). Given that this correlation is evident at the mRNA level, there may be an element of transcriptional regulation of both sirtuins involved. At the protein level however, it is notable that it is the nuclear expression of SIRT3 that correlates with the cytoplasmic expression of SIRT5, as predicted by Nakamura et al.

6.6.1.2 Indirect interactions between sirtuins

Control of common substrates by different sirtuins, and common regulators of two or more sirtuins could result in correlations between the mRNA or protein levels of those sirtuins but the relationships are likely to be weaker.

SIRT3 and SIRT1 share several substrates, and appear to have comparable effects on them, and it has already been speculated that this redundancy may offer access to and control of pools of the substrate which are required to remain separate for other reasons of cellular organisation. The correlation of mRNA levels of SIRT1 and SIRT3 may reflect linked control of these pools. The protein level data for this pairing is unfortunately not available.

SIRT3 and SIRT4 have common substrates and opposite effects on at least one of these (GDH) (Lombard, Alt et al. 2007), and a previously described and stronger mechanistic link to explain the correlation observed at the mRNA level. Of note, there is also a correlation in protein expression, but only between cytoplasmic levels of SIRT3 and SIRT4, consistent with activity in a common mitochondrial pathway.

SIRT1 and SIRT7 have opposing actions on RNAPol1, and SIRT1 and SIRT6 likewise have opposing effects on PGC1α expression. There is no correlation between either of these pairs at mRNA level, unlike the case for SIRT3 and SIRT4.

The only common regulator reported for sirtuins is the CDK1-cyclin B pathway(Grob, Roussel et al. 2009), by which SIRT2 and SIRT7 are phosphorylated. There is no observed correlation between these sirtuins at mRNA level, but there is a correlation between protein levels.

6.6.2 Sirtuin RNA Interrelationships and Sirtuin Protein Interrelationships in Normal Colorectal and Colorectal Tumour Tissue

The most striking difference between the mRNA and protein networks seen in normal and tumour tissue is the gain in complexity of the network at mRNA level, which is not translated to the protein expression level, and thus is not translated into enzymatic activity within the cell. mRNA levels are universally reduced in tumour tissue (Maxwell 2013), but it is intriguing that they show such extensive degree of inter-sirtuin correlation. An overarching control mechanism

for sirtuin transcription could be postulated, which could lose specificity in tumour tissue and act indiscriminately bringing expression of all sirtuins into line. The nature and likelihood of this mechanism is speculative.

6.6.3 Sirtuin Protein Interrelationships in Normal Colorectal Tissue and Colorectal Tumour Tissue

There is no commonality in strong correlations between normal and tumour tissue. In other words, all the sirtuin interrelationships seen in normal tissue are lost in tumour tissue, and some new interrelationships are gained.

The functional significance of these changes, the molecular interactions underlying and explaining them and the position of SIRT1 in the network are necessary for accurate interpretation of these results. However, further research into the few protein correlations which persist in this cohort of tumour tissues may help in understanding the action of sirtuins in maintaining the hallmarks of transformed cells.

Weaker correlations are generally not felt to have functional relevance. However, the presence of so many more marginal relationships between proteins in tumour cells may offer a useful picture of the breakdown of clear and precise physiological signalling into background noise.

7 Is Telomere Length in Colorectal Cancer Tissue-Specific?

Short telomeres are thought to indicate biologically aged tissue. How do we use telomere length to identify a biologically aged individual? Which telomeres should we measure? There is evidence to support the proposal that cancer occurs in more biologically aged individuals: previous work from this laboratory showed that leukocyte telomere length is shorter in individuals with cancer than in age-matched controls(Maxwell 2013).

There is evidence and mechanism to support the development of cancer in biologically aged tissue. It has also been shown that telomere length in gut tumour tissue is shorter than in adjacent non-cancerous gut mucosa(Feng, Cai et al. 2012). It is not known how telomere length in non-cancerous gut mucosa in patients with colorectal cancer relates to telomere length in gut mucosa in patients with no identified disease.

How does a biologically aged individual respond to the development of cancer? Is telomere length affected in a systemic, organism-wide manner? Is leukocyte telomere length affected specifically by the development of cancer in other tissues? The answers to these questions are not clear from published literature. In the first instance, it has not been shown whether there is a relationship between telomere length in leukocytes and telomere length in colorectal tissue in patients whether or not they have colorectal cancer.

7.1 Telomere Length in Different Tissues

Currently there is an assumption in the literature that peripheral blood leukocyte (PBL) telomere length provides an accurate surrogate measure for telomere length in other tissues. This is typified by the reference in the last paragraphs of Weischer et al's impressive study of PBL telomere length in relation to cancer risk and outcomes (Weischer, Nordestgaard et al. 2013). This is a plausible theory, as leukocytes access most parts of the body and so might be exposed to stressors throughout the body that could impact on telomere length. They might form a "lowest common denominator" for biological ageing within the body.

This would be useful in clinical terms, as leukocytes are so easily accessible and could be assessed regularly, potentially forming a valuable screening tool for age-associated disease. However to use them to screen for disease in other organs, it would be necessary to know how leukocyte telomere length related to telomere length in the tissue of interest.

7.1.1 Variation in cell turnover in different tissues

Cell turnover rates in different human tissues vary widely (from 3-7 days in GI epithelial tissues to years in neural and muscle tissues – which were initially thought to be postmitotic and virtually incapable of regeneration). Most tissues are thought to have populations of stem cells, partly differentiated cells with greater capability for replication, and capacity for unequal replication, such that they divide to form one stem cell and one cell that will not retain the higher replicative potential but merely form part of the tissue of origin, and carry out the functions of a fully differentiated cell of that tissue. The proportion of stem cells present within a tissue may reflect the replication rate, or absolute range of divisions required during the organism's usual lifespan.

7.1.2 Implications for telomere length

It is possible therefore that cell types which maintain a constant or higher rate of cell division may show more signs of ageing. This might be manifest as shorter telomeres, but this is often not the case. Faster-cycling cell compartments are postulated to maintain telomere length in stem cells in these compartments through activation of telomerase activity at the appropriate level(Chiu, Dragowska et al. 1996). It is not known whether the control of telomerase activity and telomere length is coordinated on a tissue or organism level. It is also not clear what degree of stem cell telomere length is passed on to daughter cells on asymmetric division.

7.1.3 Methodological considerations

TRFL and qPCR methods measure the average telomere length in the tissue sample. Unless specific purification steps are undertaken, samples consist of several different cell types. Within one cell type e.g. colonic mucosal cells, telomere length may vary depending on the expression of telomerase in stem

cells and partly differentiated cells. These are two potential sources of error in the measurement; they are likely to depend in part on the relevant abundance of cell types within the sample. There is limited evidence on the magnitude of the effect this has on measurements of telomere length, or of any functional effect of this degree of error.

One paper which attempted to address this problem showed robust correlations between granulocyte telomere length and mean leukocyte telomere length in 24 cases using the TRFL method and 400 cases using the FlowFISH method(Kimura, Gazitt et al. 2010). They suggested that average leukocyte telomere length is therefore a useful substitute for granulocyte telomere length without further purification measures.

An analysis of telomere length in tumour and adjacent normal tissue used colorectal tumour and sarcoma tissue (Engelhardt, Albanell et al. 1997). Light microscopy suggested assess tumour cell infiltration comprised 30% of cells viewed in colorectal tumour samples and 65% in sarcoma. TRFL analysis of the samples showed 2 distinct peaks in 17 of 51 samples and the higher peak corresponded to TRFL in the adjacent normal tissue. These peaks were hypothesized to represent populations of tumour and normal cells within the tumour specimens. Computer modelling suggested that tumour cell telomere shortening is underestimated.

Comparison of telomere lengths in selected cell populations may be possible using a combination of immunological labelling of separated cells and TRFL but this data is not available at present.

Measurement of individual telomere lengths in a cell by Q-FISH has shown that different telomeres are of different lengths even in one cell. The trigger for cellular senescence is the inability to maintain higher order telomere structure, rather than a critical number of short telomeres or a single shortest telomere length (Chin, Artandi et al. 1999; Griffith, Comeau et al. 1999; Blackburn 2000). The average telomere length in a sample is unlikely to provide a useful measurement of the likelihood of invocation of cellular senescence, but may provide an assessment of frequency of cellular senescence as it affects the whole organ.

For the purposes of this literature review, studies using FISH to measure telomere length may provide data showing correlation between telomere lengths in different tissues but the resolution of the information provided is an order of magnitude beyond that provided by mean TRFL measurements. Only 3 of the papers included in the final review used FISH methodology. They compared subsets of lingual epithelial cells, PBL and lung cells and the combination of lymphocyte, epidermal, endothelial and vascular smooth muscle cell telomere lengths.

7.1.4 Systematic review of the literature

A review of the published literature was conducted using the PRISMA methodology (Liberati, Altman et al. 2009) (see flowchart in Appendix 4) to define available information on the relationship between telomere lengths in different tissues, with especial focus on the relationship between telomere length in colorectal tissue and PBLs.

Two searches were conducted of the Pubmed database. The first used the following criteria: "Tissues"[Mesh]) AND "Telomere"[Mesh] and yielded 417 records. These were limited by the filters “English” and “Human” to 306 records. A further Pubmed search used the following criteria: "Telomere"[Mesh] AND "Cells/classification"[Mesh] and yielded 15 records. Pertinent references from bibliographies were also examined and contributed a further 30 records.

With the removal of duplicates, a total of 345 abstracts were screened. Abstracts which did not indicate or imply comparison of telomere lengths in 2 or more tissues were excluded.

84 references were collected by this means and the full text of these papers examined. The definition of ‘tissue’ was specified to include studies that measured telomere length in defined populations of cells, as demonstrable differences within subpopulations of cells would make it less reasonable to assume a link between such diverse tissues as leukocytes and gut epithelial cells. Likewise, variation within such a category as ‘leukocytes’ underlines the fact that when ‘leukocyte telomere length’ is reported, this is an average value over included subclasses of cells.

A further 49 records were excluded as they investigated telomere length in only one tissue from the same individual. Six papers were excluded because only mean telomere length for cell groups were compared statistically, rather than telomere length for cell types from one individual (Monteiro, Batliwalla et al. 1996; Kinouchi, Hiwatashi et al. 1998; Rufer, Dragowska et al. 1998; Richardson, Sverstiuk et al. 2000; Aida, Izumiyama-Shimomura et al. 2007; Romagnani, Juelke et al. 2007). These included one paper which compared rectal and caecal mucosal telomere length in 17 patients with ulcerative colitis and 17 controls with colorectal cancer(Kinouchi, Hiwatashi et al. 1998). One paper using FISH showed that telomere length profile in lymphocytes correlated with that in fibroblasts but did not address telomere length per se, and analysed only 3 cases (Graakjaer, Bischoff et al. 2003).

A total of 27 records remained which compared telomere length in 1 or more individuals, in 2 or more cell types or tissues.

7.1.4.1 Comparison articles excluded from final qualitative review

In many cases, comparison of telomere length in different tissues was not the main focus of the study and small numbers and variable statistical analysis led to the exclusion of several more papers. One study examined telomere length in ten tissues of one 72-year-old male, including blood and large intestine, and in eight tissues of a 20-week foetus, including blood but not large intestine(Butler, Tilburt et al. 1998). Although a wide range of tissues were examined, the study concentrated on only 2 individuals, and did not examine the full range of tissues in each. It concluded that there was more variation in telomere size among tissues in the adult than in the foetus, but that there was no significant difference in average telomere length of each tissue for the adult male or the foetus. Further it reported that when comparing the foetal with adult TL measurements for tissues which were available for both (blood, skin, skeletal muscle, heart, liver, pancreas, lung, brain and fat), the greatest difference between the two was seen in blood telomeric DNA.

Another study examined a range of 14 foetal tissues, not including blood(Youngren, Jeanclos et al. 1998). This study found similar telomere lengths in all tissues from the 11 individuals, but also reported significant inter-

individual variation in telomere length, and no link with gestational age. Small bowel tissue was included in the range of tissues examined from 3 cases, and colonic tissue likewise from 3 cases; 2 had both intestinal tissues available.

Analysis of endothelial cell telomere length in internal thoracic and iliac artery showed an increasing difference in mean TRFL between these two tissues with increasing age; this difference was of increasing statistical significance but considered only 4 individuals(Chang and Harley 1995).

Two studies on subclasses of leukocytes, which each reported on only 5 cases, (Yamada, Oshimi et al. 1995; Batliwalla, Damle et al. 2001), a larger study with 30 cases(Weng, Levine et al. 1995), a study of thyroid and parathyroid tissue telomere length, (Kammori, Nakamura et al. 2002) and a comparison of PBMC and fibroblasts in 3 centenarians(Mondello, Petropoulou et al. 1999) omitted formal statistical analysis. Telomere length was similar between epidermal layers in 14 individuals but the relationship was not formally analysed (Krunic, Moshir et al. 2009). Telomere lengths in 6 terminal duct lobular units in breast tissue were shorter in luminal cells than in myoepithelial cells(Meeker, Hicks et al. 2004). This pattern was not seen in the less hormonally responsive lactiferous ducts. This may reflect telomere shortening in terminal duct lobular cells due to increased number of cycles of proliferation in response to hormonal stimulation but no statistical analysis was performed.

7.1.5 Results of systematic review

The tables in Appendix 5 display data on method of telomere length measurement, tissues examined, number of cases, and age of subjects for 18 papers included in the final review. There was notable heterogeneity of study populations in terms of age, number of cases and tissues studied, and method of telomere length measurement. Two studies examined cell types purely of haematopoietic lineage, and 15 examined tissues not including those of gastrointestinal origin, leaving only 1 study which included some tissue of gastrointestinal origin.

7.1.5.1 Telomere length in haematopoietic tissues

Two studies looked at TL in subclasses of cells of haematopoietic lineage. Correlation between telomere lengths in T cell subsets was confirmed in 8 newborns, and a correlation between granulocyte and general leukocyte telomere length noted in the newborns and 2 further cohorts of 24 adults (TRFL measurement) and 400 adults (FlowFISH measurement) (Kimura, Gazitt et al. 2010). These authors also commented: "No doubt, leukocyte subsets have central and diverse roles in health and disease, but seeking to understand telomere dynamics in one type of cell vs. another is insightful only in the context of the specific hypothesis that is being tested."

In a comparison of polymorphonuclear (PMN) cells and CD34+ lymphocytes in patients with ischemic heart failure (IHF) (n=27) and controls (n=24), and their offspring (n=29 and n=25 respectively), there was a correlation between telomere length in CD34+ cells and PMN cells in controls and their offspring but not in patients with ischemic heart failure – or in their offspring(Wong, Huzen et al. 2011).

Kimura et al also provide some evidence that average leukocyte telomere length approximates granulocyte telomere length. However, in the absence of detailed information on associations with lymphocyte telomere length, there is no complete answer to the question of how (average) leukocyte telomere length might be used as a common denominator or biomarker of ageing at a wider level, particularly at the organism (or patient) level.

7.1.5.2 Telomere length in non-gastrointestinal tissues

There were 15 studies which examined telomere length in tissues including blood but excluding gastrointestinal tissues. The findings of each study are summarised in the second table in Appendix 5 and discussed here. Six studies reported on large cohorts of over 50 patients. In newborns, there was significant intra-individual correlation between each pair of mean TRF length in skin, leukocytes and umbilical artery tissue (22 skin samples - all male, 155 blood and umbilical artery samples), but a similar degree of inter-individual variability (Okuda, Bardeguez et al. 2002). Mean TRF length for skin cells was longer than for white blood cells.

A study of 62 cases showed significant correlation between telomere length in epidermis and lingual mucosa, with shorter telomeres in epidermis (Nakamura, Izumiyama-Shimomura et al. 2002).

A study of telomere length in 4 organs (heart, cerebral cortex, liver and kidney) from 100 autopsy cases (age at death 0-101 years) found significant correlations between telomere length in each tissue pair(Takubo, Izumiyama-Shimomura et al. 2002). Telomere length was longer in cerebral cortex and myocardium than in liver or kidney, and there was significant inter-individual variability as well as heterogeneity of telomere length within individual samples. There was more internal coherence in telomere length within individuals than correlation with tissue renewal rates. This was interpreted to suggest that correlations between tissue types are present and may be maintained despite different cell division rates. This is the largest study to date to examine solid organ telomere lengths but no association with blood cells was investigated.

In a study combining diabetic patients from 2 previous cohorts, 58 patients were included(Ahmad, Heraclides et al. 2012). Adjusting for age, sex and family history of diabetes, a modest correlation between relative telomere length in leukocyte and skeletal muscle was found. Given the contrasting fast and slow cycling characteristics of these cell types, this may provide evidence of an organism-wide regulation of telomere length, although the method used (like the method used in the experiments describe in this thesis) does not measure absolute telomere length.

"It is well established that TL is synchronized (positively correlated) among somatic tissues of the individual"(Aston, Hunt et al. 2012). This paper offers 3 studies in support of this claim, one of which deals with dogs, one with rhesus monkeys and one further described below, which compares telomere length in skin, blood and fat in humans. They add their own data which indicate that for 135 cases, telomere length in human sperm cells (within a protected compartment) show a moderate correlation ($r=0.33$, $p=0.0001$) with leukocyte telomere length.

In one of the most recent studies of telomere length in human tissues, 87 cases were examined (Daniali, Benetos et al. 2013). Telomere lengths in leukocytes,

skeletal muscle, skin and subcutaneous fat were measured and they reported that: "Individuals showed strong correlations of TL between tissues, such that those with long (or short) TL in one tissue typically displayed long (or short) TL in other tissues ($r = 0.72–0.84, P<0.0001$; Pearson's correlation) for each possible pairing of tissue types …. The association of leukocyte TL with the other three tissues was also consistently high ($r = 0.83–0.84$, $P<0.0001$; Pearson's correlation) for each pairing irrespective of the proliferative nature of the tissue."

Seven studies were of more moderate size. Von Zglinicki et al also found shorter telomeres in leukocytes than in fibroblasts, in a cohort of 24 patients, with a significant correlation between telomere length residuals (difference from average for that age) in PBLs and fibroblasts (von Zglinicki, Serra et al. 2000).

In a study of macaques and humans, postmitotic skeletal muscle had longer TRFL than lung or skin in both species (11 humans) (Gardner, Kimura et al. 2007). The authors noted a remarkable synchrony of different tissue telomere lengths within individuals, such that "the effect of the individual monkey or human on mean TRFL is greater than the effect of organ differences", but in humans the correlation coefficients for organ versus individual were 0.43 and 0.50 respectively. An enlarged cohort augmented with 12 autopsy cases showed 'partial synchrony' between telomere lengths in lung and skin, but this was not further analysed.

In 21 autopsy specimens, normalised telomere:centromere ratio in 3 types of lingual epithelial cells were correlated but there was no similar correlation with fibroblasts (Aida, Izumiyama-Shimomura et al. 2008). Telomere length in leukocytes correlated with telomere length in aortic wall tissue in both normal (n-12) and aneurysmal (n=20) aortas(Wilson, Herbert et al. 2008).

In 21 patients with inherited bone marrow failure disorders, blood telomere length was shorter than that measured in buccal cells or fibroblasts, and a significant correlation was observed between all pairs of tissue types (Gadalla, Cawthon et al. 2010). Each pairing of skin, blood and fat showed strong and significant correlations between telomere lengths for cohorts of 9, 9 and 15 individuals, with fat cells tending to have longest telomere length(Granick, Kimura et al. 2011).

In a cohort of 23 patients with abdominal aortic aneurysms, blood samples were available for all 23, endothelial cells and vascular smooth muscle cells for 22, and samples of epidermal cells were available for only 11 cases. Telomere length as measured by arbitrary telomere fluorescence units in each pairing of lymphocytes, epidermal cells, endothelial cells and vascular smooth muscle cells all showed strong correlations(Cafueri, Parodi et al. 2012).

Two smaller studies are included. In 9 elderly patients, significantly shorter telomere length was observed in leukocytes than in skin or synovial tissue, neither of which was significantly different from each other (Friedrich, Griese et al. 2000). There was a significant linear correlation between all pairs of tissue in this study, and it was stronger than the inverse correlation with age observed with each tissue. This was one of the first papers to propose that PBL leukocyte telomere length may provide an accessible surrogate marker for telomere length in other tissues. These data also support the theory that correlations between TL in different tissues are maintained through life, but the number of cases is small.

9 patients with interstitial pulmonary fibrosis showed a strong and significant correlation between telomere lengths measured by FISH in alveolar cells and lymphocytes(Alder, Chen et al. 2008).

7.1.5.3 Telomere length in gastrointestinal tissues

Only one study compared telomere length in large intestine with other tissues, in this case leukocytes(Craig, McKinlay et al. 2003). Telomere length was measured in samples of gastric, duodenal, colonic (from sigmoid colon) mucosa and blood by the dot blot hybridisation method. Samples from 93 upper GI tracts, and 45 colons were collected. A strong correlation was noted between the gastric and duodenal tissue telomere length, but the correlation between blood and colon was not strong (r_s = 0.25 (95% confidence interval = -0.09 to 0.54), n = 34, P = 0.1491). The correlation between blood and gastric tissue was more significant but there was no significant correlation between blood and duodenal telomere length.

7.1.6 Variable evidence of correlation between telomere length in leukocytes and somatic cells.

There was significant variation in study populations in terms of age, number of cases and tissues studied, and method of telomere length measurement. Given the heterogeneity of the studies, it is difficult to come to any conclusion regarding the usefulness or accuracy of leukocyte telomere length as a reflection of telomere length in any other tissue, and particularly colonic tissue. Most papers do not make correlation of telomeres in different tissues their main focus, but often examine this in a subset of cases and extrapolate the assumption.

The available evidence suggests that telomere length in newborns is comparable in different tissues. In adults, studies comparing subclasses of haematopoietic cells and 2 moderate sized studies comparing subtypes of epithelial and vascular cells suggested significant correlations. One of these did not show any correlation between epithelium and fibroblasts. Five large studies, five moderate sized studies and 2 smaller ones comparing relatively unrelated cell types also showed moderate and strong correlations. Ten of these included leukocytes.

Correlation of telomere lengths within tissue subtypes, or in tissues which are in anatomically close proximity is not surprising and it also appears leukocyte telomere length, in "close proximity" to all other tissues as blood cells flow throughout the body, may in fact mirror some of them. However, as Graakjaer et al comment: "Telomeres in different cell types may be subjected to different regulatory and stochastic processes both with regards to erosion and elongation. Therefore, the telomere profile found in lymphocytes may not be similar, or even present, in other cell types(Graakjaer, Bischoff et al. 2003)."

Expected variation in telomere length between tissues with a high rate of cell turnover and those with a low rate of cell turnover is also inconsistent. Telomere length varies according to a multitude of factors, which may reflect variables specific to tissue, environment and organism. Thus any effect that is demonstrable is likely to be particularly strong, but requiring such a high

threshold for activation may make telomere length a specific but not particularly sensitive test.

In the case of colorectal tissue specifically, the results obtained in the only relevant study do not support the use of leukocyte telomere length to reflect telomere length in colorectal mucosa at present.

The magnitude of inter-individual variation in telomere length is commented on in several studies and because of this, establishing normal reference ranges for telomere length would be difficult. The longitudinal studies necessary to establish the effect of individual telomere length on health outcomes directly would take a lifetime – an unfeasibly long time so far.

It has been assumed that PBL telomere length reflects telomere length in other somatic tissues; as a review of the literature has shown, there is limited evidence to prove this. A relationship has not been proven specifically between PBLs and colorectal tissue. Does PBL TL reflect colorectal tissue TL and is it shorter than in age-matched normal controls? This experiment was designed to examine the relationship between telomere lengths in three tissues from patients diagnosed with colorectal cancer.

7.2 Clinicopathological Characteristics of the Patient Cohort for Matched Samples

Sex	M	17	**Dukes' code**	NTF	1
	F	11		A	2
Age	<40	0		B	9
	40-49	2		C	11
	50-59	2		D	2
	60-69	14		Not recorded	0
	70-79	8		**Total**	**28**
	>80	2	**T stage**	NTF	0
	Total	**28**		1	0
BMI	<20	0		2	3
	20-25	8		3	19
	25.1-30	8		4	6
	30.1-35	4		Not recorded	0
	>35	6		**Total**	**28**
	Not recorded	2	**N stage**	0	11
	Total	**28**		1	9
Albumin	<35	4		2	8
	>35	24		Not recorded	0
	Not recorded	0		**Total**	**28**
	Total	**28**	**AJCC stage**	NTF	0
CRP	>10	5		1	2
	<10	23		2	9
	Not recorded	0		3	14
	Total	**28**		4	3
mGPS	0	24		Not recorded	0
	1	3		**Total**	**28**
	2	1	**Peritoneum involved**	Y	5
	Not recorded	0		N	22
	Total	**28**		**Total**	**27**
Smoker	N	17	**Margin involved**	Y	2
	Y	3		N	26
	Ex	8		**Total**	**28**
	Total	**28**	**Venous invasion**	Y	16
Tumour location	Colon	19		N	12
	Rectum	9		**Total**	**28**
	Total	**28**	**Tumour perforation**	Y	0
Differentiation	Poor	1		N	28
	Well/Mod	26		**Total**	**28**
	NTF	0	**High risk Petersen Index**	Y	4
	Not recorded	1		N	24
	Total	**28**		**Total**	**28**

Table 7.1 Clinicopathological characteristics of the patient cohort for measurement of telomere lengths in matched tissue samples.

Samples of tumour tissue, of adjacent non-malignant gut mucosa and of peripheral blood leukocytes were obtained from patients as described in the

Methods. All were analysed for relative telomere length. Clinical and pathological characteristics of this matched patient cohort (n=28) are summarised in the table above.

7.3 Comparison of Telomere Length in Peripheral Blood Leukocytes, Colorectal Mucosa and Tumour Tissue

The subset of samples of T/S in PBLs matched to tumour and normal gut samples was normally distributed, as was T/S in the sets of normal and tumour tissue samples.

Telomere length in PBLs, colorectal mucosa and colorectal tumour tissue was compared. There was no statistically significant correlation in relative telomere length between any paired combination of PBLs, normal gut tissue or gut tumour tissue.

		T/S normal gut tissue	T/S gut tumour tissue
T/S PBL	Correlation Coefficient	.078	.181
	Sig. (2-tailed)	.698	.365
	N	27	27
T/S normal gut tissue	Correlation Coefficient		.286
	Sig. (2-tailed)		.156
	N		26

Table 7.2 Results of Spearman correlations between each tissue pair. There is no significant correlation between any pair of tissue telomere lengths.

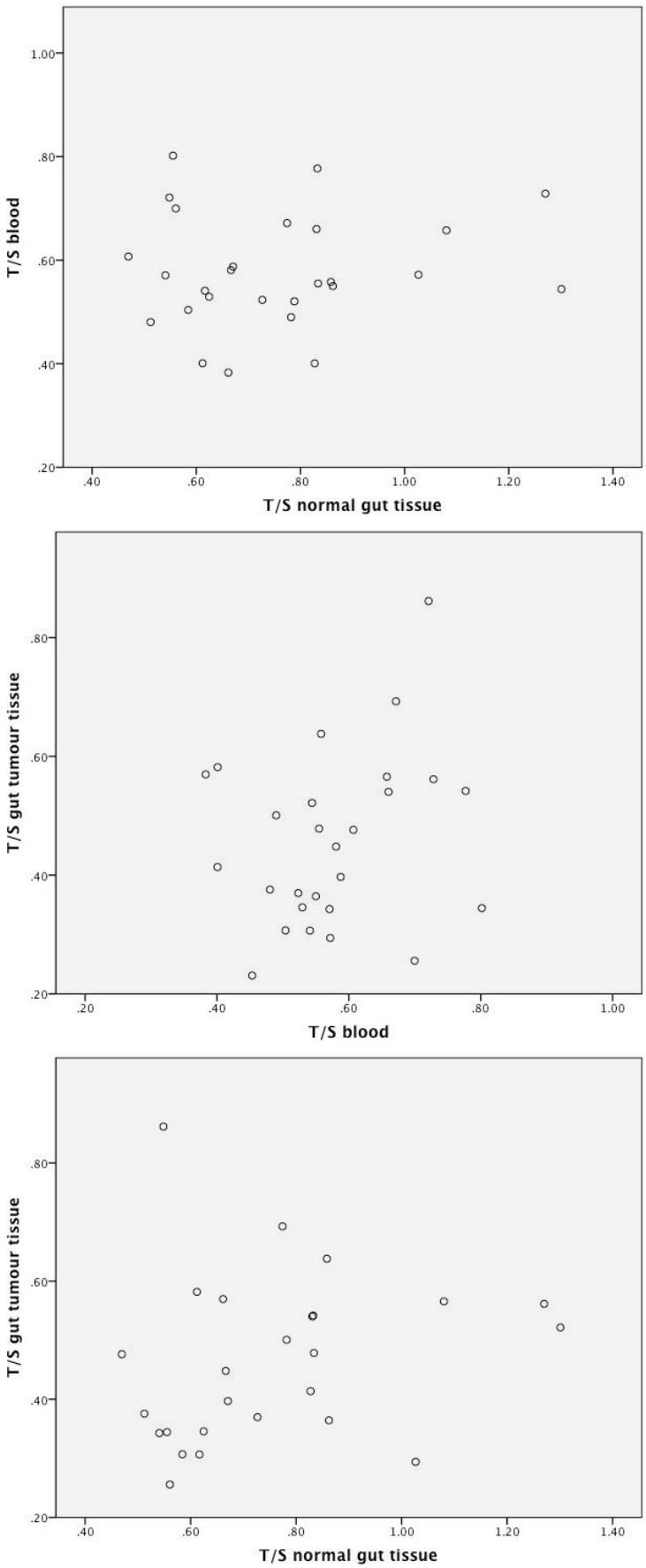

Figure 7-1 Scatterplots of tissue telomere length pairs. No significant correlations were identified.

The distribution of telomere lengths in each tissue group was examined by analysis of variance by ranks and this showed a significant difference between groups (related-samples Friedman's two-way analysis of variance by ranks, p=0.00000345). To determine the nature of the difference, this analysis was followed by related-samples Wilcoxon signed ranks tests on each tissue pair. Comparison of telomere lengths in all tissue pairs showed a significant difference in each case as tabulated below.

Tissue pair		p-value
PBL Telomeres	Normal Gut Telomeres	0.0009149575
PBL Telomeres	Tumour Telomeres	0.001517584
Normal Gut Telomeres	Tumour Telomeres	0.0000704433

Table 7.3 Significance of related-samples Wilcoxon signed ranks tests for telomere lengths in each tissue pair.

Normal gut tissue telomere length was longer than PBL telomere length and both were longer than tumour tissue telomere length: both differences are significant. These data suggest that telomere length measured from blood samples cannot provide a surrogate marker for telomere length, nor by extrapolation, an estimate of the biological age of the colorectal mucosal compartment. They suggest that PBL telomere length cannot be used to screen for biological ageing in the colorectal mucosal compartment. This contrasts with previous reports of PBL telomere length correlation with other body tissues, but is in keeping with the conclusion from the previous study comparing telomere length in blood and colonic mucosa(Craig, McKinlay et al. 2003). It is in keeping with PBL telomere length as a comparatively weak biomarker of ageing (Shiels 2010; Gingell-Littlejohn, McGuinness et al. 2013).

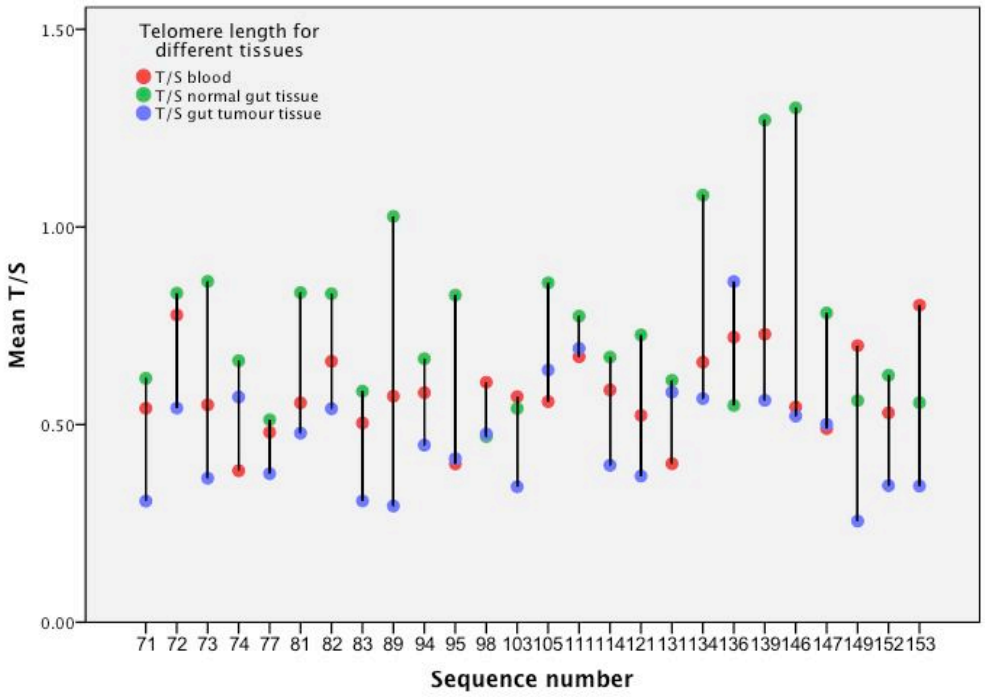

Figure 7-2 Scatterplot showing matched telomere lengths from PBL (red), normal gut (green) and colorectal tumour (blue).

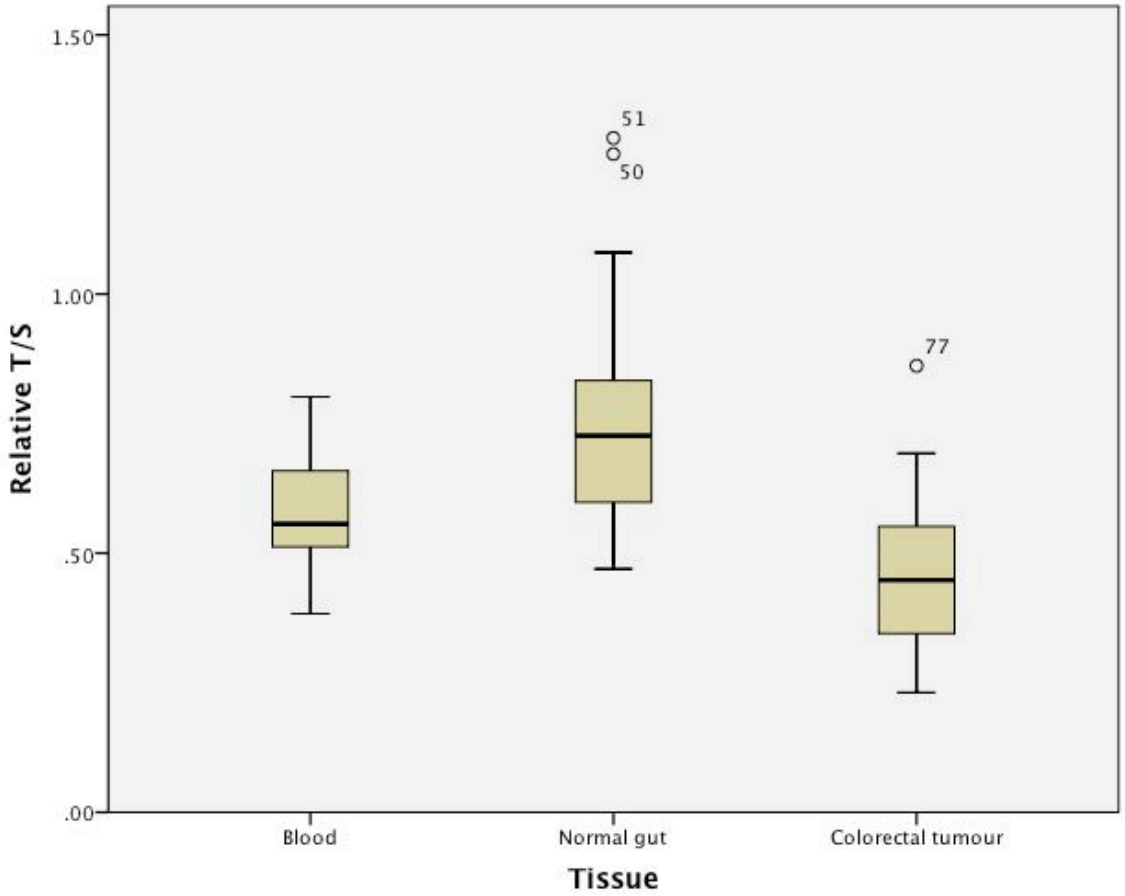

Figure 7-3 Boxplots showing distribution of relative telomere length (T/S) for each of PBLs, colorectal mucosa and colorectal tumour tissue.

7.4 Discussion

One of the aims of this project was to investigate telomere length as a useful biomarker of ageing in colorectal cancer. Cancer is an age-associated disease and so patients with cancer might be expected to show other measures of biological ageing. According to Baker and Sprott, "A biomarker of aging is 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 and Sprott 1988)

Strictly speaking, the level of function is not defined. Thus a biomarker of ageing at the level of cellular function would fulfil this definition as well as a biomarker of ageing of the whole organism. This distinction is not always made explicit in the literature, but is of crucial importance in clinical practice. The 'one measurement' that could meaningfully integrate the numerous and complex facets of ageing in one cell is a different proposition to the measurement that would encapsulate the functional frailty of an entire organism. Baker and Sprott allowed for this in the original paper, anticipating that a composite measure might be more likely. The idea of the single item biomarker was admitted to be optimistic but possible, in a review of telomere length as a biomarker for ageing and age-related disease, which focused on PBL telomere length as the 'single measurement' (von Zglinicki and Martin-Ruiz 2005). This paper quoted two of the studies outlined in the earlier literature review to support PBL telomere length as a measure of other tissues telomere length. The authors did note that much of the data is 'correlative, [and] that much of it comes from underpowered studies'.

Another review on the subject (Bekaert, De Meyer et al. 2005) acknowledged that most of available information relating telomere length to ageing was based on PBL telomere lengths as the most accessible tissue for research. There are obvious clinical advantages over sampling more inaccessible tissues which may be the actual focus of interest, but the link between PBL telomere length and telomere length in other tissues should be clarified before inference is made. Mather et al noted that the necessary prerequisite data for this assumption are not yet available.(Mather, Jorm et al. 2011)

More recently, assessment of PBL telomere length as a biomarker of ageing (BoA) at the whole organism level was examined with regard to the West of Scotland Twenty-07 Study. This study did not consider the validity of PBL telomere length as a marker of ageing in less accessible tissues. As a single item BoA, it did add predictive power over and above chronological age, but it did not account for a large proportion of the effect of chronological age, and therefore did not satisfy the Baker and Sprott defintion. Assessed as a part of a multivariate model, telomere length did add predictive power again, athough its contribution was not overwhelming.(Der, Batty et al. 2012)

The important point made by these authors is that careful thought needs to be given to the purpose of the biomarker we try to define. Do we want to predict incidence or progression of disease? Do we focus on outcome for the organism as a whole, or on the affected tissue? In the case of colorectal cancer patients, data presented here has not indicated a correlation between telomere length in blood, gut mucosa or tumour tissue. Telomere length in each compartment may be of use separately. The idea of telomere length as a biomarker of ageing might usefully be applied in several ways.

7.4.1 Telomere length as Biomarker of Ageing in Whole Organism

To accept a parameter as a biomarker of ageing of the organism, it should integrate the effects of forces which produce ageing (as an accumulation of frailty in physiological systems in an organism) in different parts of the body, eventually affecting the function of the organism as a whole. If a telomere length measurement fulfilled this criterion, it would likely be PBL telomere length. It is difficult to see how telomere length in any other tissue could integrate the effects of increasing bio-age as circulating leukocytes could.

7.4.2 Telomere length as Biomarker of Ageing in Colorectal Cancer Patients

With regard to cancer, there is evidence from this laboratory that patients with colorectal cancer have shorter PBL telomere length than age-matched controls(Maxwell 2013). In 4 case-control studies, patients with head and neck, lung, renal cell and bladder cancer had shorter lymphocyte telomere lengths than controls(Wu, Amos et al. 2003). However, the evidence of increased risk of

cancer with shorter PBL telomere length is not completely consistent. Weischer et al showed an association between PBL telomere length and cancer survival but not cancer risk in 47,102 patients with a wide range of cancers (Weischer, Nordestgaard et al. 2013). Assuming PBL telomere length in cancer patients is shorter than in age-matched controls, what does this tell us? One possibility is that these patients are more likely to develop cancer because they are more biologically aged, and PBL telomere length is a valid marker of organism bio-age.

Alternatively, these patients have short PBL telomeres because they have developed cancer. Correlative studies such as we have available at present do not offer information on causality. If we accept this second possibility, then how does cancer in the gut shorten PBL telomeres? Bekaert et al suggest a link with immunosenescence or inflamm-ageing, and there is certainly a wealth of information linking outcome with inflammatory and immune responses to the tumour (Bekaert, De Meyer et al. 2005).

The host-tumour interaction might be at the heart of the prognostic power of PBL telomeres. Rather than a direct integrative measure of agedness of different physiological systems and organs, PBL telomere length may reflect the robustness of the host systems of communication, embodied by the vascular system. Communication and integration of host defences against tumour may be the vulnerable link, as poor integration of defences may allow the tumour to gain a foothold. Defences which fail to prevent this are likely to be less competent to defend against a tumour which continues to gain in mass and genotypic advantage.

PBL telomere length may also be a biomarker of the organism's ability to cope with treatment. Where studies have shown correlation between telomere lengths in different tissues, there is some evidence that populations of cells in close physiological and anatomical proximity have more correlation in telomere length. Most of the major causes of death (non-cancer-specific, in the developed world, of aged people), are diseases of the aged vascular system (cardiovascular, cerebrovascular and chronic renal disease) or have major links with them. Respiratory disease affects the huge vascular endothelial surface area of the lungs, and type 2 diabetes is part of the metabolic syndrome, often co-existing with atherosclerosis.

Telomere length is a useful parameter. It integrates the effects of time with effects of oxidative stress, which is itself also a measure of the efficiency of cellular machinery, reflecting its functional resilience. Ageing as manifest by telomere length can indicate compartments with more 'stress', which are likely to decompensate and affect the overall body (other systems) more readily if current conditions prevail. This offers us predictive and potentially prognostic power but probably specific to the tissue of the telomere measurement. It is possible that PBL telomere length, for example, might function as a 'lowest common denominator' of biological age by reflecting non-telomere-related effects of bio-ageing in the tissues they integrate information from, but it seems reasonable to suppose that a factor that acts by affecting PBL telomere length would affect telomere length in the tissue of origin as well.

7.4.3 Telomere length as Biomarker of Ageing in Normal Colorectal Tissue: predictive or prognostic marker.

A search of the available literature has shown there is no robust evidence to support association between telomere length in PBLs and colorectal epithelial cells. No support for this assumption has been received from the results reported in this study.

There is heterogeneity of cell types in normal colonic tissue which affects the telomere length measurement made as described above. There is heterogeneity of clonal phenotypes of telomere length within a tumour. There is also a significant degree of inter-individual variation. Given these factors, one could predict that gut tissue telomere length, or tumour tissue telomere length would be difficult to use as a prognostic or predictive marker in colorectal cancer. More information is needed on change of telomere length in specific cell types over time, and with malignant transformation. With this information, in a specific individual, gut tissue telomere length may be prognostic for development of cancer. The rate of telomere change in an individual may be the critical factor for individual prognosis.

Properly powered longitudinal studies to investigate the rate of change of telomere length in colorectal tissue (and in blood) would allow us to further assess the potential usefulness of telomere length in specific tissues in colorectal

cancer. The colorectal screening program is likely to be an effective means of gaining such data. It could be useful in establishing baseline for individuals, and could be the means of monitoring deviation from it, since blood tests cannot provide an accurate assessment.

7.4.4 Telomere length in Colorectal Tumour Tissue: predictive or prognostic marker.

Gastrointestinal epithelial cells, as distinct among other types of cells in the human body, achieve the regular renewal of the gut lining by continuous replacement. In the colorectum, replacement is from the stem cell population in the intestinal crypts. Clonal popoulations in adjacent crypts originate from stem cells deep in the crypt. Colonic stem cells have come under particular interest in recent years in research on the pathogenesis of colorectal cancer because the short lifespan of non-stem gut epithelial cells is too short to allow them to develop the sort of age-associated mutations necessary for malignant transformation. Thus colonic stem cells are thought to be the source of the cancer stem cells in colorectal adenocarcinoma.

Telomere length analysis differentiates crypts which appear histologically identical,(Meeker, Hicks et al. 2004) some of which are malignant and some not. Different telomere lengths in adjacent crypts originating from stem cells which develop their own spectrum of mutations could explain the heterogeneity of telomere measurements in tumour specimens. Tumour telomere length may tell us about tumour behaviour. One paper has described an association between ratio of telomere length in colorectal tumour and adjacent normal mucosa, and survival in 147 patients(Valls, Pinol et al. 2011). This individualised result showing the effect of shorter tumour telomere length in a large study indicates the useful directions that specific tissue telomere length measurement could take. Experiments like this which associate longer telomere length with poorer prognosis suggest that individual telomere length measurements in tumour specimens could be of value in identifying tumours with increased risk of local invasion and metastasis. If these specimens were taken preoperatively, they could help tailor the use of neoadjuvant therapies. However, achieving sufficent sampling of the entire tumour to make a meaningful conclusion may be difficult.

8 Telomere length, Inflammation and Prognostic Markers in Colorectal Cancer

8.1 Introduction

Short telomeres are common in tissues which develop cancer and are postulated to reflect increased ageing and to make these tissues more susceptible to malignant transformation(Montalto, Phillips et al. 1999). The vast majority of cancer cells reactivate telomerase or show another process (alternative lengthening of telomeres - ALT) which allows them to maintain telomere length(Bechter, Zou et al. 2004). It is not clear how telomere length is controlled in transformed cells; it exhibits variation but appears to be above the critical level which normally results in cell death(Shay and Wright 2011). Cancer cells show a higher rate of joined chromosomes and break-fusion-bridge cycles of DNA damage with rearrangement of chromosomes, which are associated with dysfunctional telomeres and facilitate genomic reorganisation(Mathieu, Pirzio et al. 2004).

Telomere length is reduced in PBLs in patients with colorectal cancer with respect to age-matched controls(Maxwell 2013). Data discussed in the previous chapter indicates that telomere length in colorectal tumour tissue is reduced relative to telomere length in adjacent normal colorectal tissue. Other clinicopathological factors associated with outcome in colorectal cancer may be associated with short telomeres and offer insight into mechanisms by which short telomeres encourage malignant potential, or ways to target the interaction between telomeres and changes leading to transformation. The rationale for selection of these factors was discussed in the earlier chapter on Experimental Methods.

Data on associations between relevant clinicopathological variables and telomere length in PBLs, normal and colorectal tumour tissue are presented below.

8.2 Clinical and Pathological Characteristics of the Patient Cohort

Clinical and pathological characteristics of this patient cohort (n=95) are summarised in Chapter 3.

8.3 Overview of telomere length and clinicopathological factors

Associations between telomere lengths and 29 clinicopathological factors were examined. $p<0.01$ was accepted as the level of statistical significance; associations with p-value >0.01 are lowlighted in grey text in summary results tables.

Table 8.1 (Following page) Associations between telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue and demographic variables and classical and atypical markers of inflammation.

		PBL Telomere length	Normal colorectal tissue telomere length	Colorectal tumour tissue telomere length
Demographic variables	**Age**	Highest quartile TL is associated with higher age category, Pearson Chi square, p=0.048	Pearson Chi square, p=0.472	Pearson Chi square, p=0.616
	Sex	Male sex is associated with shorter PBL telomere length, Mann-Whitney U Test, p=0.018	Pearson Chi square, p=0.816	Pearson Chi square, p=0.345
	Weight	Pearson Chi square, p=0.573	Pearson Chi square, p=0.827	Pearson Chi square, p=0.639
	BMI	Pearson Chi square, p=0.691	Pearson Chi square, p=0.224	Pearson Chi square, p=0.691
Inflammatory markers	**WCC**	Pearson Chi square, p=0.755	Pearson Chi square, p=0.182	Pearson Chi square, p=0.863
	CRP	Pearson Chi square, p=0.131	Pearson Chi square, p=0.936	High CRP is associated with telomere length below the median, Pearson Chi square, p=0.025
	Albumin	Pearson Chi square, p=0.167	Pearson Chi square, p=0.936	Pearson Chi square, p=0.936
	mGPS	PBL telomere length varies with mGPS, Kruskal-Wallis Test, p=0.018	Pearson Chi square, p=0.511	Telomere length below the median is associated with higher mGPS. Pearson Chi square, p=0.080, Linear-by-Linear Association, p=0.039
Atypical markers of inflammation	**Hb**	Pearson Chi square, p=0.358	Telomere length in normal colorectal tissue is longer in cases with haemoglobin less than 10, Mann-Whitney U Test, p=0.034	Pearson Chi square, p=0.303
	Plt	Pearson Chi square, p=0.918	Shortest quartile telomere length is associated with platelet count above the median, Pearson Chi square, p=0.037	Shortest quartile telomere length is associated with platelet count above the median, Pearson Chi square, p=0.037
	Smoking status	Pearson Chi square, p=0.257	Pearson Chi square, p=0.177	Pearson Chi square, p=0.862

		PBL Telomere length	Normal colorectal tissue telomere length	Colorectal tumour tissue telomere length
Fetuin A		Pearson Chi square, p=0.173	Pearson Chi square, p=1.000	Highest quartile telomere length is associated with highest quartile fetuin A, Pearson Chi square, p=0.029
Serum antioxidant levels	**Lutein**	PBL telomere length below the median associates with high lutein levels, Pearson Chi square, p=0.025	Pearson Chi square, p=0.876	Pearson Chi square, p=1.000
	Lycopene	Pearson Chi square, p=0.642	Pearson Chi square, p=0.205	Pearson Chi square, p=0.302
	alpha-carotene	Pearson Chi square, p=0.552	Pearson Chi square, p=0.447	Pearson Chi square, p=0.614
	beta-carotene	Pearson Chi square, p=1.000	Pearson Chi square, p=0.833	Pearson Chi square, p=0.302
	VitA	Pearson Chi square, p=0.642	Pearson Chi square, p=0.782	Pearson Chi square, p=1.000
	VitE	Shortest quartile PBL telomere length is associated with lowest quartile Vitamin E, Pearson Chi square, p=0.040	Pearson Chi square, p=0.464	Pearson Chi square, p=1.000
Total comorbidities		Pearson Chi square, p=0.194	Pearson Chi square, p=0.420	Pearson Chi square, p=0.451

Table 8.2 Associations between telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue and fetuin A, serum antioxidant levels and comorbidities.

Table 8.3 (Following page) Associations between telomere length in PBLs, normal colorectal tissue and colorectal tumour tissue and markers of tumour-related prognostic significance.

		PBL Telomere length	Normal colorectal tissue telomere length	Colorectal tumour tissue telomere length
Tumour location		Pearson Chi square, p=0.177	Pearson Chi square, p=0.883	Shortest quartile telomere length is associated with colonic tumours, Pearson Chi square, p=0.018
Measures of cancer staging	**Dukes' stage**	Pearson Chi square, p=0.448	Pearson Chi square, p=0.271	Pearson Chi square, p=0.722
	AJCC overall stage	Pearson Chi square, p=0.411	Telomere length below the median is associated with early AJCC stage. Pearson Chi square, p=0.161, Linear-by-Linear Association, p=0.042	Pearson Chi square, p=0.557
	T stage	Pearson Chi square, p=0.352	Pearson Chi square, p=0.615	Pearson Chi square, p=0.145
	N stage	Pearson Chi square, p=0.426	Pearson Chi square, p=0.336	Pearson Chi square, p=0.549
	M stage	Highest quartile TL is associated with distant metastases, Pearson Chi square, p=0.010	Pearson Chi square, p=0.315	Pearson Chi square, p=0.077
Pathological prognostic markers	**Differentiation**	Pearson Chi square, p=0.634	Pearson Chi square, p=0.308	Pearson Chi square, p=0.308
	Margin involvement	Pearson Chi square, p=0.448	Pearson Chi square, p=0.326	Pearson Chi square, p=0.127
	Peritoneal involvement	Peritoneal involvement is associated with shorter PBL telomere length, Mann-Whitney U Test, p=0.043	Pearson Chi square, p=0.208	Peritoneal involvement is associated with shorter telomere length in colorectal tumours, Mann-Whitney U Test, p=0.015
	Tumour perforation	Tumour perforation is associated with lowest quartile PBL telomere length, Pearson Chi square, p=0.034	NO CASES	NO CASES
	Venous invasion	Pearson Chi square, p=0.841	Pearson Chi square, p=0.343	Pearson Chi square, p=0.863
	High risk Petersen index	Pearson Chi square, p=0.544	Pearson Chi square, p=0.345	Pearson Chi square, p=0.516

8.3.1 PBL telomere length and associations with clinicopathological factors

Peripheral blood leukocyte (PBL) telomere length was investigated in the full cohort of 95 colorectal cancer patients initially. The range of relative telomere length measurements was 0.605 (mean 0.615, min 0.383-max 0.988).

8.3.1.1 Demographic variables

8.3.1.1.1 Association with age

Telomere length reduces with progressive divisions in all cells as a result of the end replication problem. It is also affected in the process of ageing, as cells are exposed to other factors which cause telomere erosion. The initial assessment of telomere length therefore was to examine the relationship with age.

There was no correlation between PBL telomere length and age (see Appendix 7 for scatterplot).

8.3.1.1.2 Association with sex

Female sex is generally associated with longer telomere length but this association did not reach statistical significance in this dataset (Mann-Whitney U test, p=0.018).

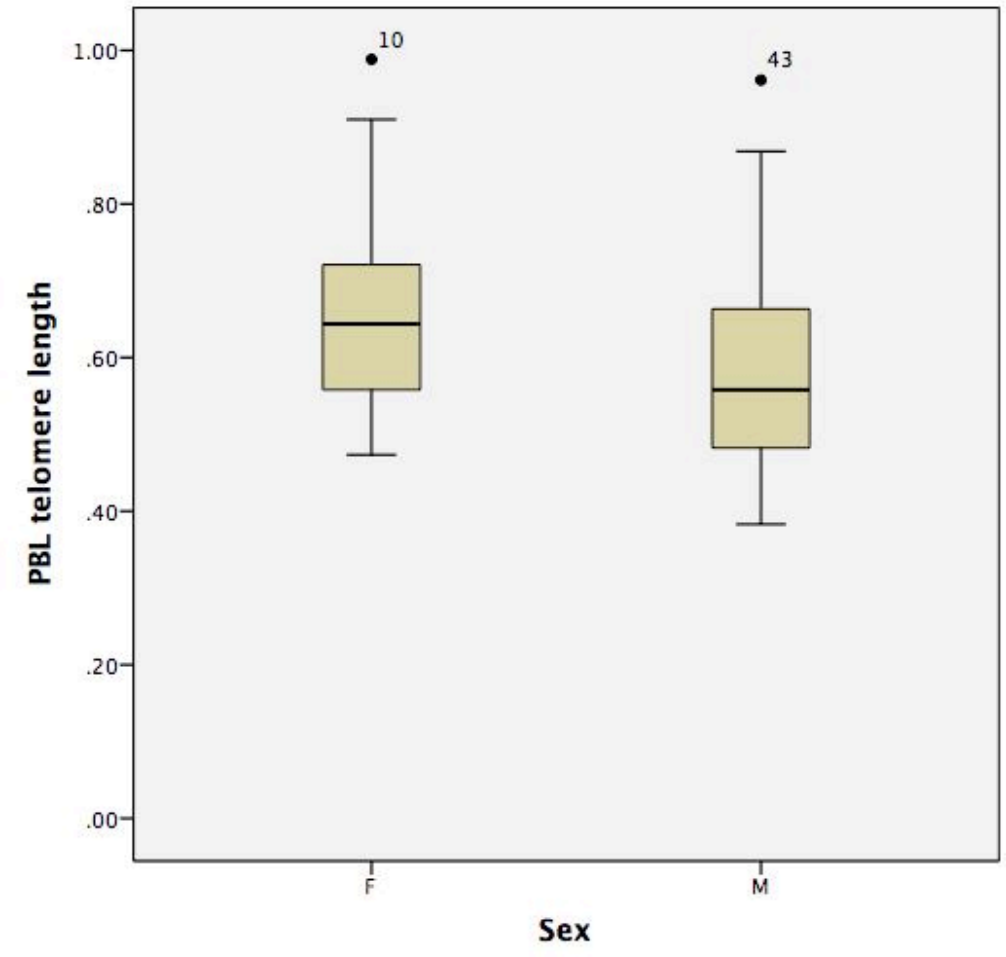

Figure 8.1 Boxplots showing telomere lengths in females and males (Mann-Whitney U test, p=0.018).

8.3.1.2 Inflammatory status

8.3.1.2.1 Associations with inflammatory markers: CRP, albumin, mGPS

There was no association between PBL telomere length and serum albumin or CRP in this cohort.

8.3.1.2.2 No association with serum fetuin A

There was no association between serum fetuin A and telomere length in PBLs in this cohort. This is contrary to a previous report in a smaller sample (Maxwell, McGlynn et al. 2011).

8.3.1.3 Measures of cancer staging

8.3.1.3.1 Association with distant metastases

In this dataset, there was no association between overall AJCC TNM stage, Dukes' stage, T stage or N stage and PBL telomere length.

An association between presence of distant metastases and highest quartile PBL telomere length did not quite reach statistical significance (Pearson Chi square, p=0.010). One might expect that if telomere length in PBLs was a measure of biological ageing, and more advanced cancer corresponded to increased biological age, any association would be in the opposite direction.

	PBL telomere length		
	Lowest three quartiles	Highest quartile	Total
No distant metastases	63	17	80
Distant metastases present	3	5	8
Total	66	22	88

Table 8.4 Longest quartile PBL telomere length is associated with presence of distant metastases (Pearson Chi square, p=0.010).

The number of cases with distant metastases was small (8/88) and an appropriately powered study would be necessary to answer this question more completely. A serological test to identify patients with distant metastases is an attractive prospect, but it is not conclusive from this data that PBL telomere length could fulfil this role.

8.3.2 Normal colorectal tissue telomere length and associations with clinicopathological factors

The range of relative telomere length measurements was 0.831 (mean 0.750, min 0.470 – max 1.301). There were no statistically significant associations with any of the clinicopathological markers examined in this cohort.

8.3.3 Colorectal tumour telomere length and significant associations with clinicopathological factors

The range of relative telomere length measurements was 0.631 (mean 0.456, min 0.231 – max 0.861). There were no statistically significant associations with any of the clinicopathological markers examined in this cohort.

8.4 Discussion

The relative telomere length to single copy gene ratio (relT/S) gives a measure of the average telomere length of cells in the sample of tissue from which the DNA was extracted. Cells were not selected for by type. Thus the average telomere length estimated is an average over all cell types present in the sample. In normal gut samples these include stromal cells and epithelial cells. In tumour samples this includes any normal cells that may remain after tumour cell infiltration and overgrowth, as well as the heterogeneous population of tumour cells. In blood, this includes all classes of leukocytes, and it is worth remembering that some inflammatory responses will disproportionately elevate different classes of leukocyte.

The telomere length measurement used was therefore not a pure analysis of gut mucosal cell telomere length or leukocyte subtype.

The number of patients for whom PBL telomere length was measured was quite small. An even smaller number of samples were available for telomere length measurement in gut mucosa and colorectal tumour tissue (n=27). The power to detect relationships with clinicopathological parameters is correspondingly affected and may be the main reason why several expected associations were not observed.

8.4.1 Telomere length and cellular vs. organism ageing

Control of telomere dynamics as a cell-specific, or cell-type specific process is readily understandable. Wider regulation of telomere length at a tissue level is possible as all cells respond to their environment and powerful homeostatic, endocrine and paracrine mechanisms operate ultimately at the molecular level on individual cells. Organism-level or system-wide effects on telomere length may be demonstrable through the competing noise of lower order level traffics described. An overarching system-wide process will also ultimately have tangible effects through events at the molecular level. Is telomere length one of the tangible effects of important processes modulating lifespan, or merely a measurable molecular-level characteristic?

Age, as such a 'system-wide' effect, does have an effect on telomere length variability in PBLs. The effect is relatively small as a proportion of total telomere length variability. Age accounts for 6-11% of total variability in telomere length in the larger studies reported, even though it is the archetypal parameter investigated(Weischer, Bojesen et al. 2012; Weischer, Nordestgaard et al. 2013). Leukocyte telomere length did not map to physical performance in a recent meta-analysis of studies (Gardner, Martin-Ruiz et al. 2013). These authors concluded that PBL telomere length may be a useful marker of cellular ageing but did not appear to offer much in assessment at a more macroscopic level. In diseases such as cancer, it may be however, that cellular ageing is the important parameter to measure as this may reflect the propensity for malignant transformation.

In this dataset, although the age range of patients for whom PBL telomere length was measured was quite wide, the overall number of cases was small. In the colorectal tissue subgroups, the age range was correspondingly narrower so that the 6-11% variability would be harder to detect.

8.4.1.1 PBL telomere length and sex.

PBL telomere length was not significantly different between males and females, in this dataset. Sex is another system-wide variable like age and, manifest as it is at chromosomal level, does appear to have demonstrable effect on PBL telomere length. The molecular mechanism underlying this effect may involve modulation of other pathways, such as the ROS handling processes.

Telomere length in different tissues is equal in males and females at birth (Youngren, Jeanclos et al. 1998; Okuda, Bardeguez et al. 2002), but in adult life, age-adjusted PBL telomere length was longer in women than in men(Jeanclos, Schork et al. 2000; Benetos, Okuda et al. 2001). These studies examined 49 twin pairs, and 193 unrelated individuals respectively, so that the cohort here may be too small to detect such differences in unmatched persons.

Women differ in the production of hydrogen peroxide from age-matched men. They also differ in the activity of antioxidant enzymes, as females have higher superoxide dismutase and catalase activity but lower glutathione peroxidase

activity.(Bolzan, Bianchi et al. 1997) The hTERT promoter contains an oestrogen-responsive element.(Kyo, Takakura et al. 1999) Telomere attrition is increased by ROS in fibroblasts (Richter and von Zglinicki 2007). Taken together these facts suggest that sex-dependent handling of reactive oxygen species may underlie the sex difference in PBL telomere length identified in the literature and replicated in this dataset.

8.4.2 Telomere length and colorectal cancer stage

To summarise, data analysis from this cohort showed longer PBL telomeres are associated with presence of metastases. Chi square analysis associated lower TNM stage with below-median telomere length in normal gut mucosa.

8.4.2.1 Telomere length in colorectal tissue and cancer stage

Several groups have investigated a relationship between cancer outcome and tumour and surrounding tissue telomere length in colorectal and other cancers. It has been proposed that critically short telomeres lead to telomere dysfunction and chromosomal instability with formation of anaphase bridges. Break-fusion-bridge cycles can produce translocations and it is proposed that these critically short telomeres and chromosomal instability underlie the transition from benign to malignant neoplasm in colonic tissue. It is suggested that telomerase is reactivated after this by established malignant cells and explains their persistence. Very long telomeres (longer than seen in normal, non-aged cells) may represent cells in which dysfunctional telomerase reactivation has surpassed levels normally observed in the tissue (Rudolph, Millard et al. 2001). This may have been the underlying process at work in the one excluded case from this cohort with extremely long colorectal tumour tissue telomere length.

In 50 human colorectal cancers and adjacent normal mucosa, longer telomeres were associated with higher levels of telomerase activity and higher Dukes' stage in colorectal tumour samples (Engelhardt, Drullinsky et al. 1997). Patients with poor survival had short telomeres in tumour samples and high telomerase activity, and patients with better survival had low telomerase activity and long telomeres. Survival was affected distinct from the accepted effect of Dukes' stage. In 57 cases of matched colorectal tumour and adjacent normal mucosa,

telomere lengths (measured using the TRFL method) between the normal and tumour tissue were correlated (Gertler, Rosenberg et al. 2004). These authors found, in keeping with the results reported in Chapter 7, that telomere length tended to be shorter in tumour tissue than normal tissue. This relationship is not absolute and although they reported a correlation between telomere length in normal and tumour tissue, tumour tissue telomere length was longer in 14% of cases.

Gertler et al also found that telomere length in TNM Stage 1 cancer was shorter than in Stage 2-4 and telomere length was correlated with overall survival. Ratio of telomere length in normal and tumour tissue was correlated with survival (RR of death=3.3 for telomere ratio >0.90). However, the association between cancer stage and telomere length in colorectal cancer is not invariable. Another study of 20 cases found a greater rate of telomere shortening in Dukes' C than in Dukes' B tumours (Kim, Kim et al. 2002). In an analysis of telomere length and telomerase activity in T cells undergoing spontaneous immortalisation, telomere length shortened through crisis, until telomerase was activated, usually some time after immortalisation(Degerman, Siwicki et al. 2010). It is possible therefore that in colorectal cancer cells, shorter telomere length in the adjacent mucosa reflects a telomere field-effect around the malignant cells which in the early stages of transformation may exhibit exceptionally short telomeres.

Telomerase activity is observed in primary CRC tumour cell lines but appears not to be constitutively maintained in invitro culture, resulting in short lifetime with limited passage; this is rescued by transfection with hTERT.(Dalerba, Guiducci et al. 2005) What maintains it in cancer cells? Is there a constant selection for telomerase-active cells? Do cells with super-active telomerase have a survival advantage, hence longer telomeres in more advanced stage cancers which have had more time to develop? In a study of 61 colorectal cancers and adjacent mucosa, there was no correlation between telomere length in tumours and telomerase activity, leading these investigators to conclude that telomerase activity may be an individual feature characteristic of a given tumour(Takagi, Kinouchi et al. 1999). Elongating telomeres whether identified by telomerase activity or elongated tumour telomeres (as compared to telomeres in background non-tumour gut epithelial cells, expressed as T/N ratio) may be the poor prognostic factor. In a group of 91 cases, there was no association between

Dukes' stage and tumour telomere length, but cases with telomere length greater than 6.12 kbp and T/N >0.66 had reduced survival.(Garcia-Aranda, de Juan et al. 2006)

It is possible that better survival in cancer reflects cells with longer telomeres ab initio in originating tissues and a cancer phenotype which does not 'over-reactivate' telomerase. In this dataset, there was a trend to a relationship between telomere length in normal gut tissue and Dukes' stage, but there was no observed relationship between stage and tumour tissue telomere length.

Many of the studies summarised are of larger size than this dataset, but nevertheless there is neither a simple correlation between tumour telomere length and cancer stage, nor any conclusion as to the correspondence or otherwise of development of malignancy, and telomere shortening or elongation. It seems clear that telomere dysfunction is associated with development of malignancy, but neither the degree nor the direction of telomere length change is explained by a simple association.

8.4.2.2 Telomere length in PBLs and cancer stage

Evidence from this laboratory has shown that colorectal cancer develops in biologically aged individuals as measured by PBL telomere length which was shorter than PBL telomere length in age-matched controls. However there is no data on mean telomere length in non-malignant colorectal tissue in patients with cancer as compared to mean telomere length in colorectal tissue in patients without cancer. Is the gut more biologically aged, or is the organism more biologically aged? Do these two factors correlate?

While there are many accounts of telomere length in tumour tissue from the colorectum and many other organs, describing a relationship or lack thereof between stage and telomere length in the tumour or adjacent normal tissue, there is no published detailed investigation of association between telomere length in PBLs and tumour stage for solid organ malignancy. In one large study of cancer risk, cancer prognosis and telomere length in PBLs, risk of death was measured in advanced (as opposed to localised) cancer as a function of telomere length(Weischer, Nordestgaard et al. 2013). They found increased risk of death

with decreasing telomere length quartiles. Previous investigation in this laboratory found no association between tumour stage and PBL telomere length in a cohort of 64 patients. This lack of information is not particularly surprising since the biological connection between the two seems tenuous.

Interpreting the finding in this cohort, of an association between PBL telomere length and presence of distant metastasis, requires caution. Presence of distant metastasis is a component of Dukes' and TNM staging, but also represents the first evidence of a cancer as a 'systemic', in the sense of 'body-wide' disease. Is that a significant difference from neoplasm as an invasive disease within the originating tissue, or from cancer which has achieved spread to nearby lymph nodes? These categorisations were developed on the basis of different clinical outcome, but may be reviewed in the era of molecular medicine. If it is a true finding, this association between PBL TL and presence of metastases implies that systemic spread of colorectal cancer manifests in systemic change in PBL telomere length.

As discussed above in the context of sex and telomere length, the effect of a systemic variable on telomere length would likely modulate stronger local forces, and may be weak but still detectable. This finding could obviously be very useful if it predicted evidence of metastatic deposits on classical imaging but this result is merely a correlation. It is interesting to speculate by what processes metastatic cancer could modulate the telomere length of circulating blood leukocytes. An underlying causative process, if it existed might tell us much of interest about the metastatic cells. Of most use would be an indication of a telomere effect prior to observed metastatic deposits. However, this association needs to be established or refuted in a properly powered study first.

It is also possible that this association is confounded. There may be a selection bias: patients with longer PBL telomeres (patients who are less biologically aged) may present with more advanced cancer. A genotype of longer telomeres in PBLs may be associated with the development of cancers that allow survival to metastasis with relatively good general health.

8.4.3 Telomere length and inflammation

There is extensive literature suggesting that inflammatory conditions of many aetiologies are associated with shorter PBL telomeres. Cancer-associated inflammation has both systemic and local aspects(Colotta, Allavena et al. 2009). These may have differential effects on 'systemic' (PBL) and 'local' (gut/tumour) telomere length. Hypoalbuminaemia and high CRP reflect activation of the systemic inflammatory response. Evidence from ulcerative colitis and other inflammatory conditions indicates that shorter PBL telomeres are associated with systemic inflammation (a manifestation of increased biological ageing). In this dataset, however, there were no significant associations between any tissue telomere length and the parameters of systemic inflammation investigated. Tumour tissue telomere length may be deregulated as part of the transformed phenotype. It is likely that the small sample size acquired here precluded detection of any relevant associations.

Weak local inflammatory response to tumour is associated with poorer survival in colorectal cancer.(Roxburgh, Richards et al. 2013) A lack of local inflammatory reaction to tumour cells could allow ongoing mutation and selection for tumour cell clones with increasingly active telomerase and longer telomeres. Longer tumour telomere lengths have been associated with poorer outcome. It is difficult to reconcile this association. Roxburgh et al reported that systemic and local inflammatory responses may be linked, with reduced local inflammatory response and high systemic response associated with poorer prognosis(Roxburgh, Salmond et al. 2009). In a recent review, Guthrie et al noted the association between tumour necrosis, decreased local inflammatory response and increased systemic inflammation, and suggest that IL-6 may be the underlying mediator (Guthrie, Roxburgh et al. 2013). IL-6 forms part of a positive feedback loop which maintains the senescence-associated secretory phenotype(Freund, Orjalo et al. 2010); a measure of local inflammation such as tumour IL-6 levels may offer further insight.

9 Concluding Discussion

The aim of this thesis was to evaluate 3 potential markers of biological ageing in the context of colorectal cancer. The data obtained is mainly descriptive but does suggest some avenues of research which are further discussed. Identification of markers of biological ageing has met with little success despite significant international investment. In part this is because it is difficult to define ageing as opposed to disease, and therefore difficult to define endpoints and deviations from what may be considered normal(Sprott 2010). For practical purposes however, markers of 'ageing and disease', where distinction between the two processes is not required may be most useful in clinical application. The subjects of study here (telomere length, fetuin A and the sirtuins) have come to the fore because of their striking effects across many disease processes which suggests a fundamental importance in the maintenance of healthy function.

Cancer is an age-associated disease but differs from other age-associated diseases. It is noted for the intrinsic growth in cell mass; there are also important changes in interaction with the host organism and in interface with and subversion of important control and signalling pathways of the organism. Although they originally came to prominence in ageing research, the three candidates investigated here are intricately linked to the cancer disease process. Further unifying features are their interactions with the inflammatory response and metabolic regulatory pathways. This underlines the dependence of cancer cells on systems such as inflammation and metabolism, as well as control of cell division, and investigation is ongoing in these areas to unravel the molecular mechanisms underpinning both these processes and their subversion in malignant transformation. New avenues of research with therapeutic intent will rely on such a nanoscale understanding of systems in the incipient era of drug design.

9.1 Telomere length in different tissues in colorectal cancer

Telomere length is most widely accepted as a potential marker of biological ageing, because of its obvious link to cycles of cell division and to the cessation of cell division. Questions remain as to how it can be interpreted to give useful information at a cellular, tissue or whole organism level. Interindividual

variability in telomere length in one tissue is large, and has hindered efforts to make generalised conclusions across a population. Interindividual variability in telomere length in one tissue may be greater than inter-tissue variability in telomere length in one individual.

One recent study measured telomere length in skin and blood and in fat and muscle and concluded that telomere length in the stem cell compartment in post-mitotic and continuously dividing tissues are equivalent(Daniali, Benetos et al. 2013). However, they did not include in this analysis the epithelial tissues which provide a large part of the human body's interface with the external environment, but which are relatively hidden. These also include the tissues which are the source of the largest non-hormone-dependent cancer burden in adults, i.e. the respiratory and gastrointestinal epithelia. The data presented in this thesis showed that, in contrast to Daniali et al's results, there was neither a correlation nor an equivalence between telomere length in two continuously dividing tissue compartments in the adult, blood and colorectal epithelium. Further large scale studies of telomere length in different tissues would be of benefit, to add a weight of evidence to one conclusion or the other. The specific tissues being compared will need to be considered as well.

9.1.1 The 'miles on the clock' hypothesis and colorectal cancer

Telomere length makes sense as marker of ageing in cells. The paracrine effect of senescent tissues, with dysregulation of handling of ROS, and the documented effect of ROS to shorten telomere length could exert a field effect in one tissue to integrate telomere length in that tissue. Telomere length could be useful as marker of ageing or of 'miles on the clock' in specific tissues. Data presented here could not determine if or how this might translate to report accurately on ageing at a higher level of organisation.

PBL telomere length as generally reported may provide information about ageing of the haematopoietic system, averaging over several cell types. Because of close proximity to the endothelial layer, PBL telomere length may give us information about the ageing of the vascular system. PBLs are somewhat unique in that they are an easily accessible source of experimental tissue which is also in indirect contact with most other tissues of the body. PBLs may integrate

information on the ROS burden of other tissues they pass through, possibly by the effect of these ROS environments on PBL telomere length. It was hoped therefore that PBL telomere length might reflect telomere length in other tissues directly enough to provide a useful surrogate, and thus enable us to draw conclusions about the robustness of the whole organism. In particular, such a measure might provide a marker of the ability of the organism, or patient, to cope with treatments with systemic effect. A specific example might be chemotherapy, as many colorectal chemotherapy regimens impose significant stress on other systems, such as the cardiovascular system.

The data reported here does not support PBL telomere length as a marker of biological ageing which can reflect the ageing of colorectal tissue presumed to be concomitant with the development of colorectal cancer, since it shows that telomere lengths in matched samples of PBLs and colorectal tissue do not correlate, and indeed appear to constitute separate populations. Telomere tissue of origin is important and it does not appear possible to use telomere lengths from different tissues as interchangeable or surrogate measures. However, telomere length in a certain tissue may still give us useful information about ageing in that tissue.

9.1.2 Potential clinical applications of telomere length analysis in colorectal cancer

In order for telomere length to function as a marker of ageing of the whole organism a unifying factor to link telomere length in all tissues would be necessary. ROS may be such a factor, but the link has not been conclusively described and may not be plausible. Nevertheless, PBL telomere length has been proposed as measure of biological ageing of the individual organism. PBL telomere length can perhaps indicate age in the vascular system; correlation with endothelial cell telomere length has already been demonstrated(Cafueri, Parodi et al. 2012). Ageing of the vascular system may have secondary effects increasing stress on other tissues through their dependence on the function of the vascular system, but one could question whether this is a directly translated relationship. However, ageing of the vascular system may have disproportionate effects on outcome from disease in any tissue, given the ubiquitous distribution of the vascular system and its secondary effects on other systems. In this sense

PBL telomere length could prove to be a reasonable single measure of ageing and outcome of disease or treatment. Although it may not be the panacea for measuring biological age of an individual, PBL telomere length may show utility in the assessment and treatment of colorectal cancer patients. Further research in telomere biology should more prominently define the tissue type under investigation than has been current practice in the past.

PBL telomere length may link to survival and capacity to cope with systemic treatments which place particular demands on the cardiovascular system, for example. Further studies to investigate PBL telomere length and survival after surgery and chemotherapy may establish it as a viable prognostic marker of 'fitness for treatment' in colorectal cancer. In the future, PBL telomere length may provide useful information on vascular risk in preoperative assessment, or prior to chemotherapy.

Telomere length in the tissue of origin of the disease studied may affect incidence and open the potential for targeted screening. Colorectal tissue telomere length has been related to progress along the neoplastic spectrum(Engelhardt, Drullinsky et al. 1997) (Nakamura, Furugori et al. 2000; Kim, Kim et al. 2002; Plentz, Wiemann et al. 2003; Feng, Cai et al. 2012) although some studies have not shown any change (Katayama, Shiota et al. 1999). It may provide early warning of increasing biological age in this tissue compartment if used in conjunction with screening programs. Large-scale validation of colorectal tissue TL in this context would be necessary and the screening programs currently in operation in the UK and elsewhere could provide a source of tissue for this purpose.

9.2 Fetuin A: Colorectal cancer and inflammation

Currently we need much more information on function of fetuin A in colorectal tissues (and in humans in general). It is a complex multifunctional protein and as such will be vulnerable to the deterioration in integration and regulation that occurs with ageing. Its role as a negative acute phase protein constitutes another pathway in the complex networks linking inflammation and tumorigenesis. Specific roles may mean it is an important part of the disease

processes in certain tissues/systems: the vascular system seems to be a prime candidate.

The evidence available demonstrated interaction with colorectal epithelial cells (Swallow, Partridge et al. 2004), with fetuin A probably functioning in the guise of TGF-β inhibitor. There is currently no data on the interaction between annexins, matrix metalloproteinases and fetuin A in colorectal cancer that is equivalent to interactions reported in breast cancer(Leite-Browning, McCawley et al. 2002; Ray, Lukyanov et al. 2003; Sakwe, Koumangoye et al. 2011; Watson, Koumangoye et al. 2012). Further work is necessary to investigate the relationship between serum levels of fetuin A and interaction with colorectal cells in normal and transformed epithelium.

Of interest, a recent publication identified fetuin A precursor protein and β-tubulin as potential diagnostic biomarkers from a case-control proteomic analysis of serum in colorectal cancer patients and healthy volunteers (Fan, Kang et al. 2014). Microtubules are formed of β-tubulin and α-tubulin heterodimers. α-tubulin is the originally identified substrate for SIRT2, which was associated with worse survival in subgroups of colorectal cancer patients in the data reported here. The interaction between SIRT2 and other members of the tubulin family will bear further investigation.

9.3 Sirtuins in colorectal cancer

The homogeneous reduction in sirtuin mRNA previously reported in colorectal cancer is not reflected in across-the-board reduction in protein expression and particularly not in protein expression in all subcellular locations(Maxwell 2013). Particularly, SIRT3 and SIRT6 protein expression in nucleus and cytoplasm are higher in tumour than in normal tissue. This indicates that there is a significant effect of post-transcriptional processing involved in sirtuin physiology. Once more were known about the most effective protein targets among the sirtuins, the corresponding post-transcriptional processing pathways could be useful therapeutic targets. It is interesting to note that SIRT3 and SIRT6 are the two members of this family shown to have an effect on longevity in mammals, but only in males (Rose, Dato et al. 2003; Kanfi, Naiman et al. 2012). It is possible that the mechanisms by which these sirtuins promote healthy chronological

ageing are also those subverted by tumour cells to maintain their own proliferative potential. Given the sex-differences in the effect of these sirtuins some interaction with oestrogen signalling is a logical assumption, and further research into this link may also impinge on the interaction between fetuin A and its differential effects on the sexes.

9.3.1 Sirtuins and the MTR hypothesis

This study supports the MTR hypothesis by confirming expected protein expression of the sirtuins with some caveats. Cytoplasmic staining cannot be directly assumed to be mitochondrial, and nuclear staining does not necessarily imply telomeric interaction. This study also adds complexity to the picture by widening the distribution of sirtuin localisation, particularly the pool of 'nuclear' sirtuins. A higher definition representation of the function of sirtuins to localise protein expression precisely within both nucleus and cytoplasm would be necessary to provide even stronger support for the MTR hypothesis.

9.3.2 Sirtuins and inflammation

Broadly speaking, data presented here indicated higher SIRT2 nuclear staining was associated with worse survival, higher levels of systemic inflammation and worse prognostic features on pathological assessment of the tumour. Lowest quartile levels of SIRT2 in the nucleus may also have a detrimental effect given the association with poor survival, but this appears to have less widespread ramifications among other relevant clinicopathological parameters.

Higher nuclear staining for SIRT3 was associated with venous invasion. Lower cytoplasmic staining was linked to more systemic inflammation and poor differentiation, so that the balance between nuclear and cytoplasmic localisation seems important, and a higher cytoplasmic proportion may be favourable.

Higher SIRT4 and SIRT5 cytoplasmic staining was associated with reduced systemic inflammation, and nuclear staining for both is a novel finding. Higher SIRT6 cytoplasmic and nuclear staining were associated with less systemic inflammation. Pathological markers of poor prognosis were associated with the opposite cytoplasmic staining.

High nuclear and low cytoplasmic expression of SIRT7 was associated with systemic inflammation. High nuclear staining was also associated with several pathological markers of poor prognosis.

From the recap given above, higher nuclear staining associates with increased systemic inflammation and with poor pathological prognostic features, almost across the board, in keeping with the established interaction between cancer and inflammation. High cytoplasmic staining seems to be more specifically associated with systemic inflammation, and again this is true of all the sirtuins except SIRT2.

Comprehensive comparison of sirtuin expression in other disease processes may help distinguish where sirtuins fit in the inflammatory response, and whether this is altered in cancer. Modulation of the inflammatory system and the immune response is emerging as a potent therapeutic option in cancer as in metabolic and vascular disease, but it will be important to fully understand the ramifications of such treatment in all these conditions in order to avoid significant unwanted effects(Stunkel and Campbell 2011). Understanding of the intricacies of the physiological responses is a fundamental prerequisite.

9.3.3 The absent factor: SIRT1

SIRT1 is the human sirtuin about which most is known, as it has been most widely studied and is most similar to the yeast sirtuin which sparked the initial interest in this diverse family of enzymes. Absence of data from this cohort on SIRT1 staining in colorectal cancer is a significant deficit. It also leaves a measureable gap in conclusions that may be drawn on sirtuin interrelationships. However, this deficit will hopefully be remedied in the near future as a suitable antibody to SIRT1 is optimised.

In the meantime it is worth noting that in a tissue microarray containing normal colonic mucosa, adenomas and colorectal cancers of various stages stained for SIRT1 higher staining was found in the base of crypts where active cell division might be expected (Kabra, Li et al. 2009). Increased staining in all cells with adenomatous morphology was observed and staining in colorectal cancer cases was very variable but appeared to reduce in later stage cancers. This is in

keeping with a variable benefit of SIRT1 expression which changes with the developing genetic and intracellular milieu of a progressive cancer phenotype.

9.4 Systems biology approach to colorectal cancer

There is a sea-change under way in the scientific approach away from the reductionist dissection of molecular pathways, which has characterised the investigative focus for many decades. The reductionist approach is an essential starting point and is central in clarifying the detail of networks. More recently, however the limits of this approach have been appreciated and it is recognised that to fully understand how a network functions requires an appreciation of the spatiotemporal context as well as knowledge of the precise interactions of its physical components.

Systems biology is the study of component parts within the environment where they function. Ageing has already been recognised as a good exemplar for the application of the principles of this discipline(Kriete, Sokhansanj et al. 2006). In order to achieve full understanding of a process which is initiated by repair events at a molecular level but whose effects are evident at a macroscopic level, it is necessary to integrate all the observations and all the theories about ageing into a coherent whole. Systems biology moves the emphasis from a 'competing models' way of explaining observations to an integrative approach which starts simply and adds layers of complexity as the answers unfold.

In taking advantage of all the tools and approaches available, it is recognised that these will span many fields and necessitate increasingly interdisciplinary modes of working.

Systems biology theory proposes that the connectedness of a node within the network is more important than any single effect (or for example, the absolute concentration of a serum protein). Modulation of a well-connected node would be a more efficient and efficacious intervention, but it is necessary to understand all the ramifications - i.e. all about the network - to anticipate all effects, and particularly if therapies are designed to modulate it, to minimize unwanted effects.

Sirtuins and fetuin A both have connectivity in several diverse fields of research indicating that they might be promising “well-connected” players in the ageing process. The findings presented here support their importance and indicate that further study in these fields is likely to be useful.

Many molecules have been identified as tumour suppressor or oncogene, or as protective or tumorigenic in the cancer literature. Although these are useful ideas, they can be limiting, particularly as the nature of many signals can change depending on time, location and milieu – the spatiotemporal context. The network view which maps interactions without loaded effect descriptions (‘value judgments’) may be more useful.

Cells have evolved many defences against the proliferation of damaged genotypes, but cancer cells develop because they happen to undergo a combination of alterations and deteriorations which allow them to circumvent all of these defences. Only a few of the myriad combinations of random alterations produce cells with the potential to survive the breakdown of the machinery that normally integrates them into the whole organism. There may even be a required order to these changes, or required sequences or motifs within the random succession of changes to avoid cell death along the sequence.

In studying the networks involved in cancer biology, the aim is to identify, from the myriad of changes in ageing cells, those alterations in cellular function that are key to development of the malignant phenotype – the ‘tipping points’ in development of cancer. Computer modelling of networks constructed from experimental data may help decipher the critical paths among a plethora of associations(August and Koeppl 2012; De Ambrosi, Barla et al. 2013).

The ‘Swiss cheese model’ of error is one which may be relevant to cancer biology(Reason 2000). This is a construct applicable to many types of systems: inflight controls, hospital patient safety systems, molecular control of ROS sequestration and signalling. In any complex system, there are many layers of control designed to prevent calamity, none of which is perfect, but which together provide overlapping safety nets which usually compensate for the inevitable holes in each net. However, sometimes ‘the holes line up’, several safety nets fail, and the situation escalates from there. If the situation in

question is the development of a cancer cell, the Swiss cheese model maps very well to the multi-hit model of cancer development, and the adenoma-carcinoma sequence in colorectal cancer.

Networks will be vulnerable where they lack redundancy and at the highest levels of control, where any malfunction has multiple downstream effects. These are likely to be the location of tipping points in cancer development. There is an extensive field of computational modelling and system analysis already reaching maturity which is being harnessed to attack the conundrums of cancer(Shen, Goonesekere et al. 2012; Yun, Johnson et al. 2012; Drier, Sheffer et al. 2013; Schweiger, Hussong et al. 2013). Some of the most forward thinking approaches involve experts in non-traditional disciplines and it will be exciting to see how their approach can shed new light on difficult problems(Fillon 2013).

9.4.1 Sirtuin Interrelationships: network construction

None of the sirtuins studied can be firmly identified as a biomarker of ageing, although all seem to be intricately involved in the cellular dynamics of colorectal cancer. One demonstration of this, and of the importance of networks in further understanding the molecular basis of malignant transformation and its defining characteristics, is in the change in the sirtuin interrelationships in normal and tumour tissue. Although the correlations between protein expression used to build the networks do not imply causation, or direct interaction, it is likely that correlated proteins act in the same pathways and so such correlations may be used as starting points to unravel the specific interacting components.

9.5 Ageing and disease are not synonymous

Ageing and disease are not synonymous. ‘Healthy ageing’ with retention of most faculties and functions is possible and indeed is the holy grail of medicine. Disease can occur in younger people, sometimes due to a relatively few defects which by their effect at a whole organism level highlight their importance within the system. Ageing meets disease when the cumulative effect of frailty (progressive minor deteriorations) happens to include a few defects which are of greater consequence. The outcome of disease in conjunction with ageing is more serious because the frailty (multiple minor deteriorations) of the individual

means that systems which normally prevent escalation of a problem lack the resilience to do so.

Disease in young people highlights tipping points – important wayposts in the development of the disease where the outcome of an event (a genetic mutation, or a protein accumulation, for instance) drastically changes the downstream course. These events are identifiable because the negative outcome is of sufficient importance as to overcome the resilience of a younger system, with its inbuilt checks and balances more likely to be intact than in an older individual.

9.6 Ageing is fundamentally a breakdown in integration

All pathways interconnect, all processes are under integrated control, in the homeostatic regulation of the internal milieu of an organism. This is a basic tenet of life and the living organism. Ageing is a breakdown of integration, between cells, and between component pathways within a cell. Successful defence against threats to an organism presuppose an intact response pathway but with age, none of the 'normal' responses can be assured and as such the pathologies of age are those of an infinite permutation and perturbation of the evolutionarily designed order within cell biology.

Ageing is a stochastic process but only a tiny minority of aged cells are able to escape integration in the organism and persist as cancer cells. What are the controllers of integration? These will be subject to ageing (deterioration) like any other system or components, but the deterioration in their function will have wider reaching consequences. Systems biology points to the most connected nodes in a network as being the most important, those with the most effects, the most control and influence.

The systems biology approach is therefore an intrinsically sensible one for the study of ageing. It aims to examine, define and understand the multilevel, hierarchical structure of the networks that underlie and form the organism. It will also eventually allow us to model perturbations to the system and examine the outcomes on different levels as well. In the meantime however, it provides a useful focus on the importance of networks.

9.7 Limitations of this work, and future directions

This study was designed to produce a multifaceted assessment of several potential markers of biological ageing in the specific context of colorectal cancer, and to describe any interactions between them. For this reason, ideally, all measurements would have been made on matched tissue, for more powerful direct comparisons. Thus integration of telomere length measurements, fetuin A and colorectal tissue sirtuin protein expression awaits further research.

9.7.1 Telomere length

The major limitation of the telomere analysis study was the small number of colorectal tissue samples available. Although this study did show a significant difference between telomere length in PBLs and colorectal tissue, with no correlation between the two, small numbers of tissue samples limited the investigation of relationships between colorectal tissue and tumour tissue telomere length and clinicopathological parameters. The small number of tissue samples obtainable was directly related to practical aspects of the study. Incidence of colorectal cancer during the study period was outwith experimental control although the catchment group of hospitals was widened to try to mitigate this effect. Tissue collection from patients who did develop colorectal cancer required a separate consent process that was not under the purview of this team, and took second place to the demands of clinical practice. These problems could be overcome for future research by extending the timescale of the study, and by dialogue to integrate the research process more closely with the function of the clinical team.

As a proof of concept study, however, it has been shown that with good communication between clinical and laboratory teams, it would be reasonable to continue the collaboration and gain sufficient power to generate clinically relevant data. This could be used to determine the validity of colorectal tissue telomere length as a potential early marker of tissue ageing and increased risk of dysplasia and cancer. This may be of particular use in the context of colorectal screening for high-risk patients.

9.7.2 Fetuin A

Fetuin A is a serum protein which interacts with tissues via cell surface receptors. Binding of fetuin A is reduced in tumour tissue compared to normal tissue (Swallow, Partridge et al. 2004). It would have been very informative to correlate fetuin A tissue binding with serum fetuin A levels, by construction of a TMA using samples from patients from whom serum had also been sampled in the preoperative period.

Staining of the sirtuin TMA for fetuin A would be one relatively straightforward additional integrative study to assess any associations between fetuin A and sirtuins or any effect of fetuin A staining and survival. Such data would also be useful to corroborate the only existing data on fetuin A binding to colorectal tumour(Swallow, Partridge et al. 2004).(Condeelis and Pollard 2006)

9.7.3 Sirtuin immunohistochemistry

The obvious deficit in the immunohistochemical data available, namely the lack of data on expression of SIRT1 protein has been mentioned earlier. Although the immunohistochemical study of sirtuin expression in colorectal cancer which forms a large part of this thesis has been very informative, it would also be ideal to integrate it with analysis of telomere length and serum fetuin A levels, from one cohort.

The advantage of using the TMA was obviously in the large number of cases to which it gave access, allowing relatively powerful analysis. However, the normal cohort for comparison numbered only 20 cases. Given that much of the data on sirtuin protein expression in normal colorectal tissue is lacking, it would have been useful to have a larger normal cohort for robust baseline comparison with the tumour tissue. No definitive conclusion could be drawn on possible alteration in expression of SIRT6 or SIRT7 in tumour tissue with respect to normal colorectal tissue because of this lack of power. Equally, information on baseline relations between sirtuin expression in normal tissue and clinicopathological factors such as serum inflammatory markers is lacking in the reported literature.

It is always challenging to coordinate the collection of matched samples of different tissues from one patient as it necessitates liaison between clinical and laboratory-based teams, and must be streamlined to fit in with the patient's

medical treatment which is the first priority. As the importance of gaining a complete snapshot of the biological system becomes established, integration of the laboratory and clinical teams may become more widespread, and will undoubtedly be easier as familiarity between them grows.

9.8 Biomarker of ageing: a panel rather than a key factor.

Although only a preliminary study of any biological networks is attempted in this thesis, it is clear that the processes involved are of a considerable degree of complexity. It is inherently unlikely that a single unifying Biomarker of Ageing could capture a summary of their state in a meaningful manner. Certainly, none of the candidates studied here has that power. CDKN2A is one biomarker which is showing promise and has prognostic value in colorectal cancer as well but further study is necessary to clarify its place (Shiels 2012; Xing, Cai et al. 2013).

In the future, it is most likely that, once sufficient information was gathered and tailored to an individual, computer modelling techniques may be able to identify networks or parts thereof that were under particular strain. Analogous to the tissue-monitoring macrophages(Condeelis and Pollard 2006), they could direct treatment to tissues under stress, ideally before significant organ senescence and damage had occurred.

9.8.1 Composite biomarkers of ageing and disease in colorectal cancer

The key attribute of useful biomarkers is prognostic or predictive value. Although the data reported here comes from 2 separate cohorts, these cohorts are of similar populations i.e. both unselected patients with colorectal cancer from Glasgow hospitals separated by a short time interval. The same analysis was performed in respect of the accepted prognostic markers of colorectal cancer examined. Some of the experimental variables (like SIRT2 nuclear expression) appear to be clear candidates for further research and may have some therapeutic potential.

As previously noted however, biomarkers of ageing and disease may be of use individually or in a composite panel.

9.8.2 Composite biomarkers of cancer stage

TNM stage is the strongest prognostic indicator in colorectal cancer. The cohorts in this study are limited in that they had very few patients with metastatic cancer at the time of sampling. For this reason, no meaningful analysis of associations with M stage are made. Associations between T stage and N stage parallel and explain the associations with Dukes' stage, which is therefore not discussed further.

9.8.2.1 T stage

Of the experimental variables assessed, lower serum fetuin A, was associated with higher T stage and there were trends towards associations with higher SIRT2 and SIRT7 nuclear, and SIRT3 cytoplasmic staining. Individual underlying mechanisms have been discussed in earlier chapters and there does seem to be a biologically plausible explanation for most of these associations. It would be interesting to analyse this collection of markers further in a single cohort. This may identify confounding factors for instance between fetuin A and the sirtuins. Equally, however, they may each add predictive value. Of significant interest also would be an analysis of fetuin A in serum and in association with tumour tissue. This would require a large longitudinal study. Very small amounts of tissue are required, and it would be useful to establish baseline measurements in normal tissue. As mentioned in regard to future research on telomere length, the colorectal screening program could be a source of such tissue, with matched serum samples from appropriately consented patients at the time of screening colonoscopy.

9.8.2.2 N stage

Reduced nuclear expression of SIRT7 associated with N stage, with a trend to association with reduced nuclear expression of SIRT6 and enhanced nuclear expression of SIRT2. Large scale analysis of this panel as described above could guide adjuvant chemotherapy if this combination was proven to add predictive value to results obtained from pathological examination of an operative specimen. Analysis of diagnostic biopsies preoperatively might in the future identify a cohort of patient who could benefit from neoadjuvant chemotherapy to increase the chance of a curative resection.

9.8.2.3 Differentiation

Differentiation parameters are somewhat subjective, although this may change as more molecular markers are identified, but is a well-recognised poor prognostic indicator. SIRT3 nuclear staining, in addition to the variables associated with high T stage completes the panel of markers which may offer useful information on the molecular mechanisms which produce poorly differentiated cells. Poorly differentiated cancers tend to be more locally invasive, and these markers may go some way to explaining this.

9.8.2.4 Venous invasion

Venous invasion as well as N stage is an indicator of systemic spread with a significant effect on prognosis. It is also even more difficult to detect on imaging and sometimes on superficial biopsies preoperatively so that predictive markers may be of great use. Increased nuclear staining (of SIRT3, SIRT4, SIRT6 and SIRT7) and SIRT4 cytoplasmic expression are prominent.

While these combinations of sirtuins and sometimes fetuin A would be interesting to investigate further, it must be remembered that higher levels of tumour protein expression are associated with pathological markers of poor prognosis, this is against a background of reduced expression relative to normal. Establishment of normal baselines in a larger cohort may allow a proper assessment of the relevance of these observations.

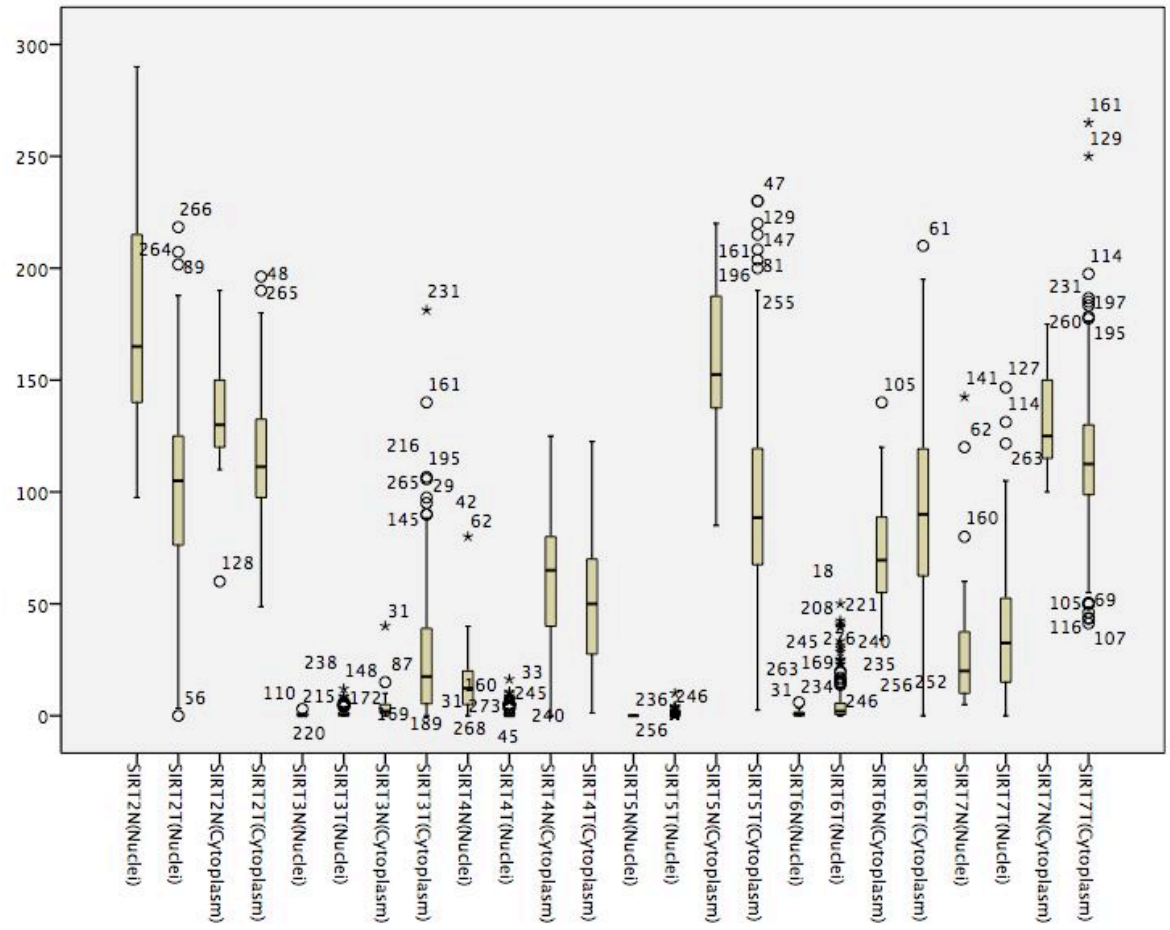

Figure 9.1 Boxplots showing Histoscores for nuclear and cytoplasmic expression of SIRT2-SIRT7 in normal and tumour tissue. The suffix N or T after SIRTX denotes normal or tumour tissue.

9.9 Frailty not ageing or longevity?

As translational science in many branches of medicine becomes more of an interdisciplinary routine, shared and standardised terminology will become more valued and familiar.

9.9.1 Frailty

Frailty is a clinical syndrome usefully applied to give an overview of the whole patient, which is gaining ground in the clinical arena(Chen, Mao et al. 2014). It encompasses several of the functional measures of bioageing such as gait, grip strength and physical exhaustion. The concept of frailty is readily applicable to the study of systems in many disciplines and as such offers a common readily understood language in which to communicate on this subject. It embodies the concept of agedness, without reference to chronology. It implies loss of resilience, loss of the capacity to effect adequate repair, and loss of the

integrity of systems and structures that are the consequence of ageing at any level.

Hayflick has said with some truth that ‘The fundamental ageing process is not a disease but it increases vulnerability to disease’. Frailty may be the best way to describe this nexus of age and disease in a way that addresses the issues of importance to patients and clinicians, in terminology which can also be used by molecular biologists, genetic engineers and other involved professions. Assessment of frailty is already in use to predict postoperative morbidity in patients undergoing surgery for colorectal cancer(Tan, Kawamura et al. 2012).

Resilience is the hallmark of youth; frailty may be the defining characteristic of ageing and the most valuable target for combating age-associated disease.

9.9.2 Measurement of ageing: different levels

The meaningful measurement of ageing, or measurement of frailty, depends on the level of organisation under consideration when we make the measurement. Thus, measurement of frailty of molecular systems involves specific assessment of the function of elements of molecular machinery, such as enzyme activity. In different circumstances, measurement of organ function may be more clinically useful. For some purposes, social functioning of a patient may be the relevant parameter to assess. All of these examples are used in the evaluation of a patient with cancer, and future research will need to develop assays tailored appropriately.

10 Appendices

10.1 Appendix 1: Telomere and 36B4 primers for the QPCR reaction using the Roche LightCycler 480

10.1.1 Telomere and 36B4 Primers

Telo 1 Sequence (5' to 3'):

CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT

Telo 2 Sequence (5' to 3'):

GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT

36B4d Sequence (5' to 3'):

CCC ATT CTA TCA TCA ACG GGT ACA A

36b4u Sequence (5' to 3'):

CAG CAA GTG GGA AGG TGT AAT CC

10.2 Appendix 2: Telomere and 36B4 plate running conditions for the QPCR reaction using Roche LightCycler 480

10.2.1 Telomere plate running conditions (Roche Light Cycler LC480)

Program Name	Heat Start (HS)			
Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:10:00	4.4	
Program Name	Amplification (Amp)			
Cycles	30	Analysis Mode	Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	4.4	
59	None	00:00:10	2.2	
72	Single	00:02:00	4.4	
Program Name	Melt			
Cycles	1	Analysis Mode	Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:30	4.4	
65	None	00:00:30	2.2	
95	Continuous	00:02:00	0.29	2
Program Name	Cool			
Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
40	None	00:00:10	2.2	

Table 10.1 Telomere plate running conditions

10.2.2 36B4 plate running conditions (Roche Light Cycler LC480)

Program Name	Heat Start (HS)			
Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:05:00	4.4	
Program Name	Amplification (Amp)			
Cycles	30	Analysis Mode	Quantification	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	4.4	
58	None	00:00:15	2.2	
72	Single	00:00:15	4.4	
Program Name	Melt			
Cycles	1	Analysis Mode	Melting Curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
95	None	00:00:30	4.4	
65	None	00:00:30	2.2	
95	Continuous	00:02:00	0.29	2
Program Name	Cool			
Cycles	1	Analysis Mode	None	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)
40	None	00:00:10	2.2	

Table 10.2 36B4 plate running conditions

10.3 Appendix 3: Layout of slides for JP-CRC-TMA 1-5

Layouts are shown on the following page.

17	Lung	Liver	Kidney	Heart	Prosta	Pancre	Colon												
16																			
15	1	1	1	1	2	2	2	2	3	3	3	3	4	4	4	4	5	5	5	5
14	6	6	6	6	7	7	7	7	8	8	8	8	9	9	9	9	10	10	10	10
13	11	11	11	11	12	12	12	12	13	13	13	13	14	14	14	14	15	15	15	15
12																				
11	16	16	16	16	17	17	17	17	18	18	18	18	19	19	19	19	20	20	20	20
10	21	21	21	21	22	22	22	22	23	23	23	23	24	24	24	24	25	25	25	25
9	26	26	26	26	27	27	27	27	28	28	28	28	29	29	29	29	30	30	30	30
8	31	31	31	31	32	32	32	32	33	33	33	33	34	34	34	34	35	35	35	35
7	36	36	36	36	37	37	37	37	38	38	38	38	39	39	39	39	40	40	40	40
6																				
5	41	41	41	41	42	42	42	42	43	43	43	43	44	44	44	44	45	45	45	45
4	46	46	46	46	47	47	47	47	48	48	48	48	49	49	49	49	50	50	50	50
3	51	51	51	51	52	52	52	52	53	53	53	53	54	54	54	54	55	55	55	55
2	56	56	56	56	57	57	57	57	58	58	58	58	59	59	59	59	60	60	60	60
1	61	61	61	61	62	62	62	62	63	63	63	63	64	64	64	64	65	65	65	65
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Table 10.3 Layout of JP-CRC-TMA 1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
17	Lung	Liver	Kidney	Heart		Prosta	Pancre	Colon														
16																						
15	66	66	66	66		67	67	67	67	68	68	68	68		69	69	69	69	70	70	70	70
14	71	71	71	71		72	72	72	72	73	73	73	73		74	74	74	74	75	75	75	75
13	76	76	76	76		77	77	77	77	78	78	78	78		79	79	79	79	80	80	80	80
12																						
11	81	81	81	81		82	82	82	82	83	83	83	83		84	84	84	84	85	85	85	85
10	86	86	86	86		87	87	87	87	88	88	88	88		89	89	89	89	90	90	90	90
9	91	91	91	91		92	92	92	92	93	93	93	93		94	94	94	94	95	95	95	95
8	96	96	96	96		97	97	97	97	98	98	98	98		99	99	99	99	100	100	100	100
7	101	101	101	101		102	102	102	102	103	103	103	103		104	104	104	104	105	105	105	105
6																						
5	106	106	106	106		107	107	107	107	108	108	108	108		109	109	109	109	110	110	110	110
4	111	111	111	111		112	112	112	112	113	113	113	113		114	114	114	114	115	115	115	115
3	116	116	116	116		117	117	117	117	118	118	118	118		119	119	119	119	120	120	120	120
2	121	121	121	121		122	122	122	122	123	123	123	123		124	124	124	124	125	125	125	125
1	126	126	126	126		127	127	127	127	128	128	128	128		129	129	129	129	130	130	130	130
0																						

Table 10.4 Layout of JP-CRC-TMA 2

17	Lung	Liver	Kidney	Heart		Prosta	Pancre	Colon												
16																				
15	131	131	131	131		132	132	132	132	133	133	133	133		135	135	135	135	136	136	136	136
14	137	137	137	137		138	138	138	138	139	139	139	139		140	140	140	140	141	141	141	141
13	142	142	142	142		143	143	143	143	144	144	144	144		145	145	145	145	146	146	146	146
12																				
11	147	147	147	147		148	148	148	148	149	149	149	149		150	150	150	150	151	151	151	151
10	152	152	152	152		153	153	153	153	154	154	154	154		155	155	155	155	156	156	156	156
9	157	157	157	157		158	158	158	158	159	159	159	159		160	160	160	160	161	161	161	161
8	162	162	162	162		163	163	163	163	164	164	164	164		165	165	165	165	166	166	166	166
7	167	167	167	167		168	168	168	168	169	169	169	169		170	170	170	170	171	171	171	171
6																				
5	172	172	172	172		173	173	173	173	174	174	174	174		175	175	175	175	176	176	176	176
4	177	177	177	177		178	178	178	178	180	180	180	180		181	181	181	181	182	182	182	182
3	183	183	183	183		184	184	184	184	185	185	185	185		186	186	186	186	187	187	187	187
2	188	188	188	188		189	189	189	189	190	190	190	190		191	191	191	191	192	192	192	192
1	193	193	193	193		194	194	194	194	195	195	195	195		196	196	196	196	197	197	197	197
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Table 10.5 Layout of JP-CRC-TMA 3

17	Lung	Liver	Kidney	Heart		Prosta	Pancre	Colon											
16																			
15	198	198	198	198		199	199	199	199	200	200	200	200	201	201	201	201	202	202	202	202
14	203	203	203	203		204	204	204	204	205	205	205	205	206	206	206	206	207	207	207	207
13	208	208	208	208		209	209	209	209	210	210	210	210	211	211	211	211	212	212	212	212
12																			
11	213	213	213	213		214	214	214	214	215	215	215	215	216	216	216	216	217	217	217	217
10	218	218	218	218		219	219	219	219	220	220	220	220	221	221	221	221	222	222	222	222
9	223	223	223	223		224	224	224	224	225	225	225	225	226	226	226	226	227	227	227	227
8	229	229	229	229		230	230	230	230	231	231	231	231	232	232	232	232	233	233	233	233
7	234	234	234	234		235	235	235	235	236	236	236	236	237	237	237	237	238	238	238	238
6																			
5	239	239	239	239		240	240	240	240	241	241	241	241	242	242	242	242	243	243	243	243
4	244	244	244	244		245	245	245	245	246	246	246	246	247	247	247	247	248	248	248	248
3	249	249	249	249		250	250	250	250	251	251	251	251	252	252	252	252	253	253	253	253
2	254	254	254	254		255	255	255	255	256	256	256	256	257	257	257	257	258	258	258	258
1	259	259	259	259		260	260	260	260	261	261	261	261	262	262	262	262	263	263	263	263
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Table 10.6 Layout of JP-CRC-TMA 4

17	Lung	Liver	Kidney	Heart	Prosta	Pancre	Colon												
16																			
15	264	264	264	264	265	265	265	265	266	266	266	266	267	267	267	267	268	268	268	268
14	269	269	269	269	270	270	270	270	271	271	271	271	272	272	272	272	273	273	273	273
13	274	274	274	274	275	275	275	275	276	276	276	276	17 N	17 N	27 N	27 N	39 N	39 N	40 N	40 N
12																			
11	62 N	62 N	87 N	87 N	90 N	90 N	105 N	105 N	109 N	109 N	110 N	110 N	114 N	114 N	125 N	125 N	128 N	128 N	139 N	139 N
10	141 N	141 N	160 N	160 N	182 N	182 N	224 N	224 N	232 N	232 N	1 N	1 N	31 N	31 N	81 N	81 N	97 N	97 N	131 N	131 N
9	147 N	147 N																		
8																				
7																				
6																				
5																				
4																				
3																				
2																				
1																				
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Table 10.7 Layout of JP-CRC-TMA 5

10.4 Appendix 4: PRISMA flowchart for systematic review of literature on telomere length in matched tissues

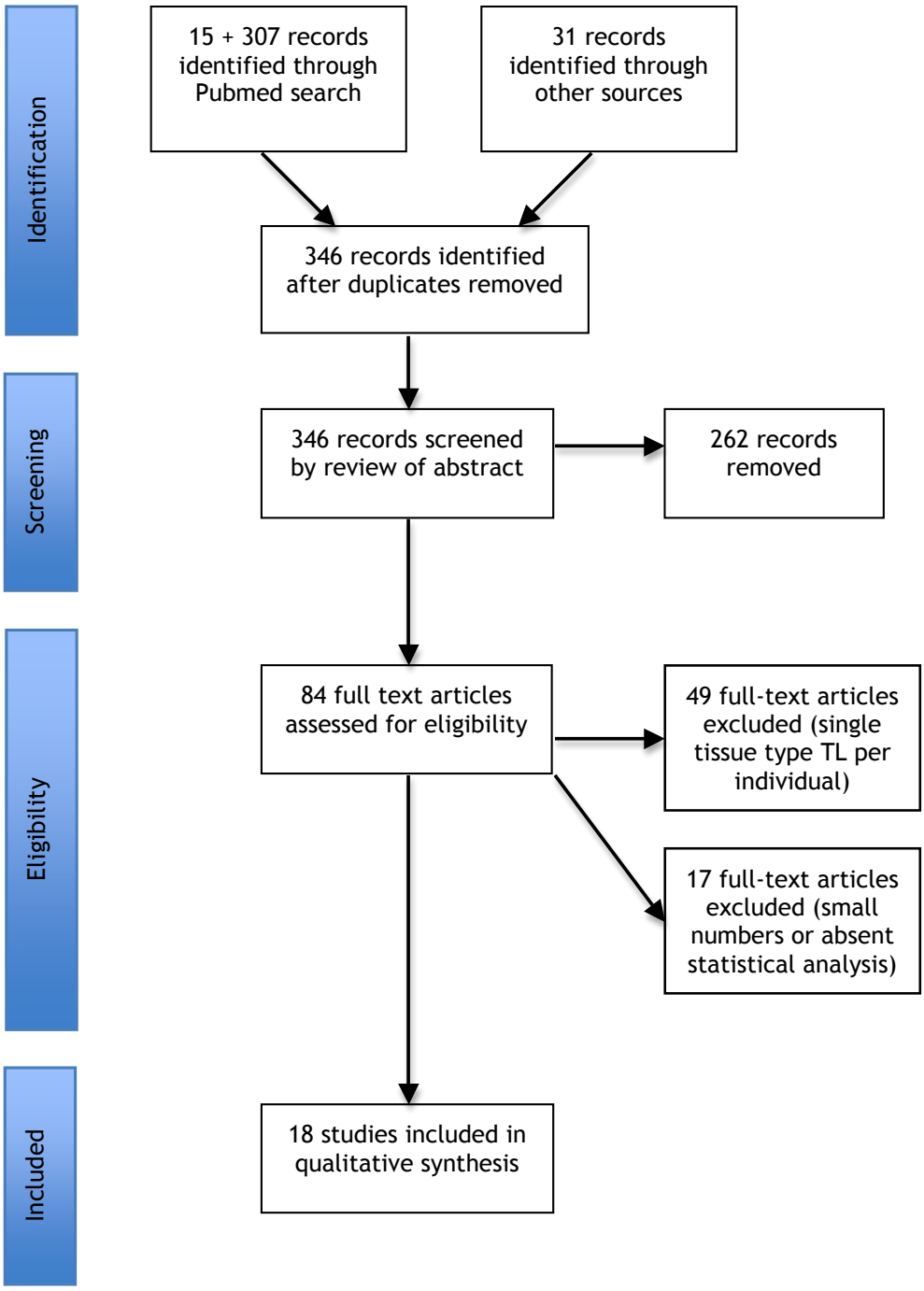

Figure 10.1 PRISMA flowchart applied to systematic review of the literature on telomere length in matched tissues.

10.5 Appendix 5: Literature comparing telomere lengths in different cell types

Tables summarising the literature considered in the qualitative review are on following page.

Pub year	Journal	First author	Pubmed number	Telomere length measurement method	Tissue types included	Number of cases	Age range (yrs)
2011	PLoS ONE	Wong	21876736	qPCR	CD34+ lypmohcytes and mononuclear cells	27 CHF pts/ 29 controls	62-76
2010	Experimental Hematology	Kimura	20600576	TRFL Southern blot and flow FISH	Peripheral blood leukocytes and subclasses	8 24 400	0 22-34, 0-100

Table 10.8 Two papers comparing telomere length in classes of haematopoietic cells

Table 10.9 (And following pages) Papers comparing telomere length in non-gastrointestinal tissues

Pub year	Journal	First author	Pubmed number	Telomere length measurement method	Tissue types	Number of cases	Age range of cases
2000	Mechanisms of Ageing and Development	Friedrich	11080530	TRFL Southern blot	Leukocytes Skin Synovial tissue	9	73-95
2000	Laboratory Investigation	von Zglinicki	11092534	TRFL Southern blot	Leukocytes Fibroblasts	24	28-91
2002	Pediatric Research	Okuda	12193671	TRFL Southern blot	WBC Umbilical artery Foreskin	165 158 23	newborn
2002	J Investigative Dermatology	Nakamura	12445186	TRFL Southern blot	Epidermis Lingual epithelium	62	0-101
2002	Experimental Gerontology	Takubo	11830355	TRFL Southern blot	Cerebral cortex Myocardium Liver Kidney Spleen	137 168 191 137 30	0-104, few subj 10-40 yrs
2007	J Gerontology Series A	Gardner	17452729	qPCR	Lung Serratus anterior Skin	11+12 autopsy 11 11+12 autopsy	50-76

Pub year	Journal	First author	Pubmed number	Telomere length measurement method	Tissue types	Number of cases	Age range of cases
2008	PNAS	Alder	18753630	FISH	Leukocytes Lung alveolar cells	9	Not recorded in paper
2008	European Heart Journal	Wilson	18762552	qPCR	Leukocytes Aortic wall	12 autopsy, 20 AAA patients	56-78
2008	Experimental Gerontology	Aida	18590810	QFISH: normalised telomere: centromere ratio	Lingual cells: basal Lingual cells: parabasal Lingual cells: prickle cells Lingual cells: fibroblasts	21 autopsy specimens	0-101
2010	Aging	Gadalla	21113082	QPCR	Leukocytes Buccal Fibroblast	21 inherited bone marrow failure	8-43 years
2011	Eplasty	Granick	21436892	TRFL Southern blot	Leukocytes Fat Skin	16	14-58
2012	Diabetic Medicine	Ahmad	22747879	qPCR	Leukocytes Muscle	58	34-62
2012	Molecular Human Reproduction	Aston	22782639	TRFL Southern blot	Leukocytes Sperm	135	18-68
2012	PLoS ONE	Cafueri	22514726	QFISH	Lymphocytes Epidermal cells Endothelial cells Vascular smooth muscle cells	23 11 21 21	61-78
2013	Nature Communications	Daniali	23511462	TRFL	Leukocytes Skeletal muscle Skin Subcutaneous fat	87	19-77

Pub year	Journal	First author	Pubmed number	Telomere length measurement method	Tissue types included	Number of cases		Age range (yrs)
2003	European J Gastroenter- ology & Hepatology	Craig	14560153	Dot blot hybridisation Telo/genomic DNA	Duodenal cap Gastric antrum Sigmoid colon Blood	90 34	86 90	20-90

Table 10.10 Single paper comparing telomere length in gastrointestinal tissues and others.

10.6 Appendix 6: Inter-rater and intra-rater correlations for Histoscores

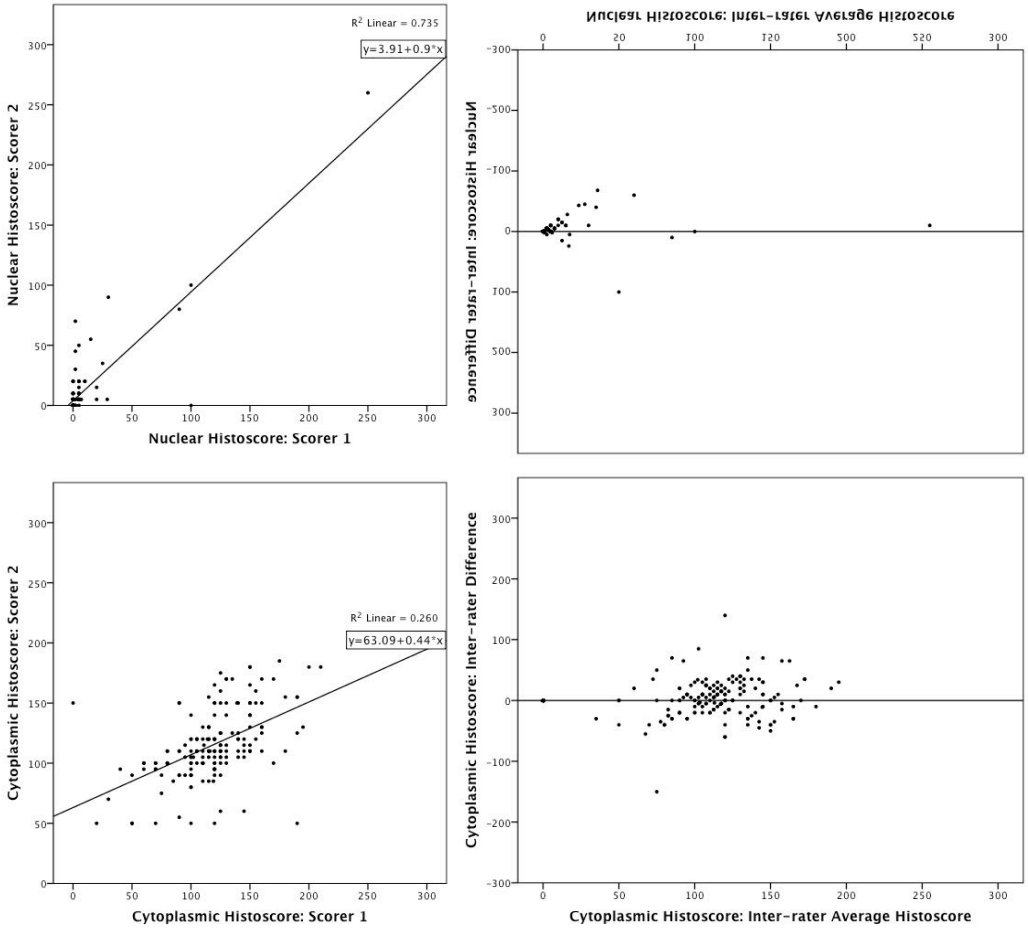

Figure 10.2 Scatterplots and Bland-Altman plots for inter-rater nuclear and cytoplamic Histoscore correlations. ICCC for nuclear Histoscores was 0.851 and for cytoplasmic Histoscores was 0.913.

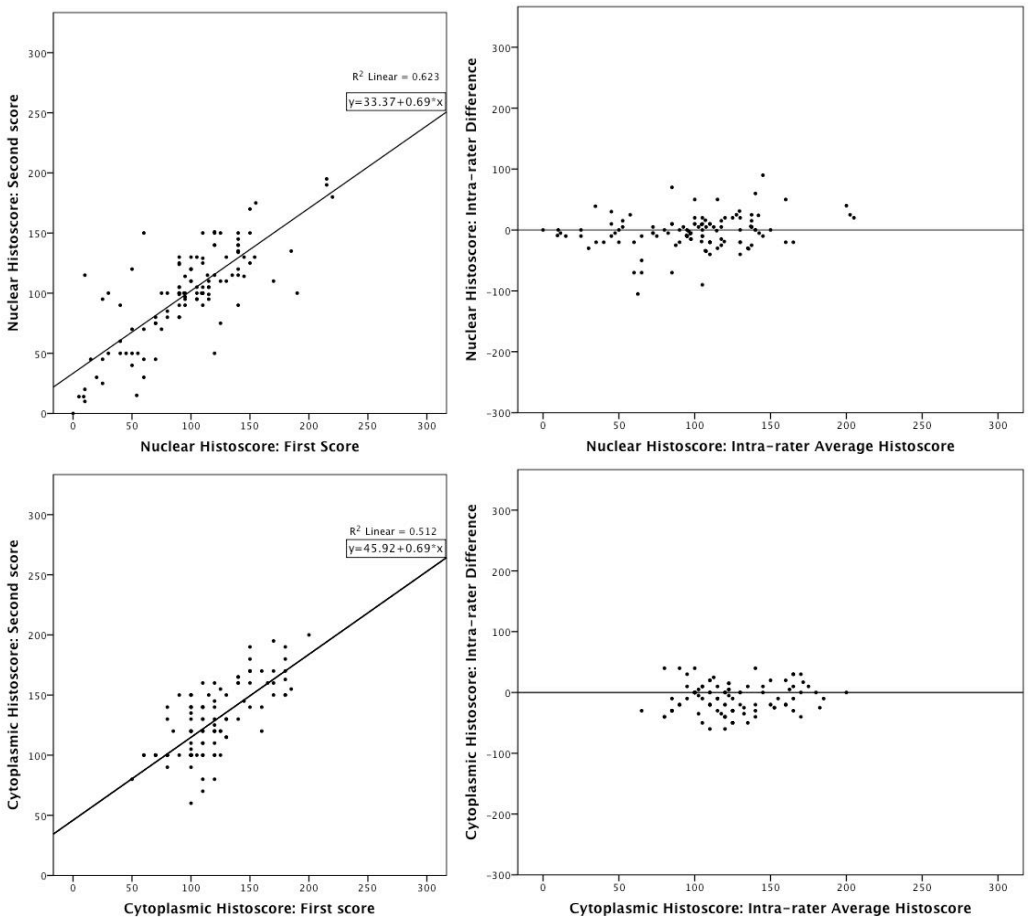

Figure 10.3 Scatterplots and Bland-Altman plots for intra-rater nuclear and cytoplamic Histoscore correlations. ICCC for nuclear Histoscores was 0.789 and for cytoplasmic Histoscores was 0.716.

10.7 Appendix 7: PBL telomere length and age

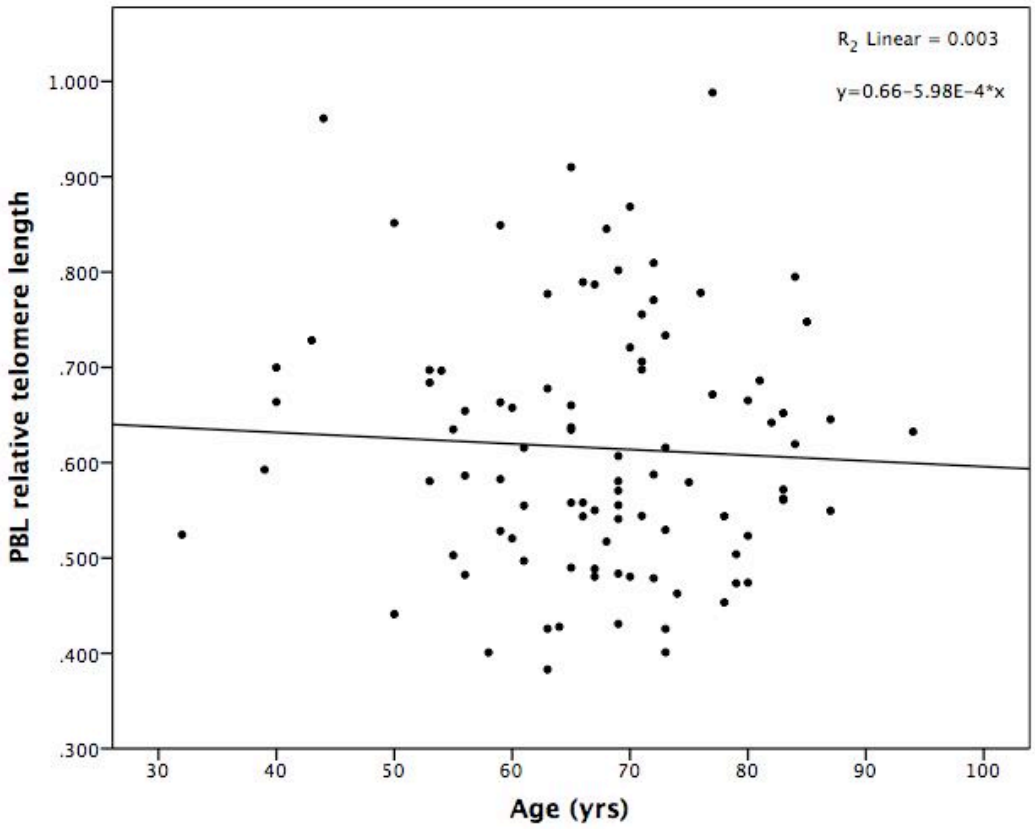

Figure 10.4 Scatterplot of age vs PBL telomere length with line of best fit (Rsq =0.003). The correlation is weak and not statistically significant.

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