INVESTIGATIONS INTO THE EXPRESSION OF SIRTUINS IN BREAST CANCER: *IN VIVO* AND *IN VITRO* STUDIES

BY

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Summary of Thesis.

Breast cancer remains the most common malignancy in women and a major cause of morbidity and mortality in the western World, despite the advances in diagnosis and treatment. The main challenge remains to identify new, and improve existing treatment modalities. Understanding the mechanism by which tumours grow and metastasise is key to developing new therapeutic targets

Similar to most cancers, the incidence of breast cancer increases with age. Therefore, genes involved in biological ageing and factors affecting genomic integrity, considered critical to cellular senescence and organismal life span, are also relevant to neoplastic transformation and tumour growth. Thus exploring factors associated with biological ageing in cancer may improve our understanding of the disease as an aberration of normal biological ageing and result in new prognostic markers or therapeutic targets. There is increasing evidence for the involvement of sirtuins in biological ageing, along with other essential cellular processes including cell cycle control, DNA damage repair and differentiation. This is suggestive of a possible role for sirtuins in cancer. Therefore, this study was conducted to investigate a potential role for sirtuins in breast cancer disease (including anti-tumour treatment).

Firstly, Real time PCR was used to compare the transcriptional expression level of individual sirtuin genes in vivo. The experimental result showed that only SIRT1 and SIRT4 showed an association with age in "normal patients" (normal and non malignant patient grouped together), with decreasing levels of SIRT1 and increasing levels of SIRT4 being associated with increasing chronological age. All sirtuin levels were significantly decreased in malignant tumours, when compared to either normal and/ or non-malignant biopsies. Decreased relative transcriptional expression of SIRT1, SIRT2, SIRT3, SIRT6 and SIRT7 showed significant association with higher tumour grade, when breast cancer patients were divided according to the known histopathological markers.

The Kaplan-Meier analysis for cancer specific survival and tumour recurrence was preformed on entire patient cohorts and in patient subgroups selected to have moderate prognosis (ER^{+ve} and NPI between 3.4 and 5.4). The Kaplan-Meier survival analysis showed that higher levels of SIRT6 and SRT7 were associated with a longer survival period in all patient cohorts. Furthermore, higher levels of SIRT6 remained significantly associated with better survival, when breast cancer patients were selected to have

intermediate prognosis (ER^{+ve} and NPI between 3.4 and 5.4). Whereas, higher levels of SIRT7 remained significantly associated with longer survival period only in patients with ER^{+ve} tumours. The Kaplan-Meier analysis showed that lower levels of SIRT1 gene expression were associated with longer patient survival and lower tumour recurrence in a patient group, selected by NPI, to have an intermediate clinical prognosis. Multivariate Cox-regression analysis demonstrated that the relative transcriptional level of the SIRT6 gene was independent of tumour size, grade, nodal status, oestrogen receptor status, lymphovascular invasion status, and the NPI in influencing survival.

The decreased sirtuin gene expression observed in this study is consistent with an anti-cancer effect and suggests that sirtuins might be implicated in breast cancer pathogenesis. For example, decreased levels of SIRT2 might assist DNA-damaged tumour cells, as indicated by the decreased expression of another sirtuin involved in DNA damage responses, SIRT6, in escaping cell cycle arrest during tumour initiation and progression. Furthermore, The associations between sirtuins and survival period suggest that these sirtuins (especially SIRT6) might be used as an additional prognostic marker in breast cancer patients, especially in those individuals who have equivocal prognostic pathological markers. Therefore, the level of expression of sirtuin genes (SIRT6) might help explaining those breast cancer cases, which behave unexpectedly, according to the known pathological prognostic markers.

Secondly, The changes in the relative transcriptional expression levels of the sirtuin genes were investigated in response to adjuvant chemotherapy therapies, commonly used in breast cancer (Tamoxifen and Docetaxel), in breast caner cell lines. The first experiment determining sirtuins changes in response to Docetaxel treatment for 72 hours in ER^{-ve} breast cancer cell line (MDA-MB-231) showed significant increase in the relative transcriptional expression levels of all sirtuins after Docetaxel treatment. These data were consistent with the pro-apoptotic role for SIRT1, SIRT3 and SIRT7, and suggestive of DNA damage involvement at higher doses of Docetaxel, as indicated by increased SIRT6 and XRCC5. Finally, increased SIRT2 levels are suggestive of SIRT2 involvement in the mitotic arrest caused by Docetaxel, through its contribution to microtubule dysfunction.

The second experiment, determining sirtuin changes in response to Tamoxifen treatment in ER^{+ve} (MCF-7) and ER^{-ve} (MDA-MB-453) breast cancer cell lines, showed significant increase in the relative transcriptional expression levels of all sirtuins after Tamoxifen treatment. These data were consistent with the pro-apoptotic role for sirtuins. Furthermore, the observed increased levels of SIRT6 are suggestive of DNA damage involvement at

higher doses of Tamoxifen. Another noteworthy result of this experiment is the increased levels of SIRT2 in response to Tamoxifen treatment. This might explain the failure of a TAM-treated cell to proceed through the cell cycle, in spite of the increases in transcription factors that promote cell cycle after Tamoxifen treatment. There was no significant difference in sirtuin changes after Tamoxifen treatment between these two cell lines to indicate that sirtuin changes were ER-dependent.

In total, the data accumulated from this study demonstrated the involvement of sirtuins in breast cancer disease (pathogenesis and anti-tumour treatment) and suggest the possible use of SIRT6 as a novel, additional and biological prognostic marker. Finally, this study suggests that sirtuins activators, rather than inhibitors, might be beneficial in breast cancer disease and enhance the response to adjuvant chemotherapy.

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Author's Declaration

I declare that the work presented in this thesis has been carried out solely by myself, except where indicated below.

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List of Abbreviation

ALT	Alternative Lengthening of Telomeres
ATM	Ataxia Telangiectasia Mutated
BAE	Base Excision Repair
BAX	Bcl2 Associated Protein
Bcl2	Bcl-2 (B-cell lymphoma 2)
CBP	CREB-Binding Protein
CDC14B	Dual specificity protein phosphatase
CDKI-2A	Cyclin-Dependent Kinase Inhibitor 2 A
cIAP 2	Inhibitor of Apoptosis Protein 2
COUP-TF	Chicken Ovalbumin Upstream Promoter Transcription Factor
CTIP	COUP-TF- Interacting Protein 2, CTIP-2
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyriboNucleotide TriPhosphate
DTT	DiThioThreitol
ER	Oestrogen Receptor status
ER+ve	Oestrogen Receptor positive
ER-ve	Oestrogen Receptor negative
FHL2	Four and a Half LIM domains protein 2
FOB	replication Fork Block Protein
FOXO1	Forkhead Box O1
G	Tumour Grade
GCIP	GRB2 Cycline Dependent Interacting Protein
GRB2	Growth Factor Receptor-Bound protein 2
Hey 1	Hairy/Enhancer-of-split related with YRPW motif protein 1
HOXA 10	Homeobox Transcription Factor 10
L	Liter
LPA	Lysophosphatidic Acid
LV	Lymphovascular Invasion Status
LV+ve	Lymphovascular invasion Positive
LV-ve	Lymphovascular Invasion Negative
MDM2	Murine Double Minute 2
MEF	Mouse Embryonic Fibroblasts
mGPS	modified Glasgow Prognostic Score
MHC	Myosin Heavy Chain
N	Nodal Status
N+ve	Node Positive
NAC	No Amplification Control
NET	NeuroEpithelial cell Transforming Gene
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NPI	Nottingham Prognostic Index
NTC	No Template Control
N-ve	Node Negative
PCAF	p300/CBP-Associated Factor
PCNA	Proliferating Cell Nuclear Antigen
PCR2	Polycomb Repressive Complex 2
PGC-1a	Peroxisome roliferator-activated receptor Gamma Coactivator 1-alpha
PNC1	Pyrazinamidase/ Nicotinamidase 1
Polβ	Polymerase β
PUMA	p53 Upregulated Modulator of Apoptosis
Rb	RetinoBlastoma
RNA	RiboNucleic Acid
RT	Reverse Transcribe
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis,
shRNA	Short Hairpin RNA
SIR	Silent Infromatin Regulators
siRNA	Small Interference RNA (siRNA)
SIRT	Sirtuins
T	Tumour Size
Tms	Melting Temperatures
TTBS	Tris Buffered Saline-Tween
µl	Micro Liter

Chapter I

Introduction

1.1 Introduction.

Breast cancer is the most prevalent female cancer globally and its incidence is increasing in the western world. Breast cancer is the second most common cancer in the UK, with over 44,000 new cases diagnosed every year and 14,500 deaths attributed to this disease annually. Breast cancer accounts for one in three of all female cancers and is second to lung cancer in causing female-cancer specific deaths. Breast cancer represents a heterogeneous group of diseases that display a wide spectrum of behaviour ranging from slow growing, non-metastatic disease with good prognosis through to fast growing disease with poor prognosis (metastasis and short survival). Despite the large number of breast cancer studies that have been carried out, the underlying molecular changes involved are still not fully understood. Identification of novel biological changes associated with this disease may improve our understanding and result in new prognostic markers, or therapeutic targets being identified. Given the close relationship between the incidence of cancer and chronological age, malfunctions of genes involved in biological ageing and factors involved in maintaining genomic integrity, have been hypothesised to be involved in tumourigenesis (1, 2). These include the BRCA genes, which have a role in DNA double strand break repair (3-6).

Other potential candidate genes include Sirtuins. These comprise a gene family phylogenetically conserved from bacteria to man, with known involvement in ageing and DNA damage repair. First described in yeast, the Silent Information Regulator 2 (SIR2) gene product was demonstrated to function as an NAD+-dependent histone deacetylase (7). SIR proteins have subsequently been described in diverse cellular processes, including regulation of gene silencing at telomeres and mating type loci, DNA repair, structural and functional maintenance of the nucleolus, regulation of the mitotic cell cycle, meiosis and ageing (8-11). In humans, seven ortholouges, were identified and termed sirtuins (SIRT1-7). These will be presented in detail in the following sections.

The study of breast cancer as an aberration of normal biological ageing may provide an alternative platform for understanding the disease. Specifically, a better understanding of the molecular processes involved in sensing, assessing and signalling cellular damage might be informative in such a context (2). This might be alternative to the traditional study of breast cancer biology that focuses on malignancy transformation as a result of abnormalities in normal growth and proliferation. Therefore, better understanding of the

ageing process and genes involved in biological ageing and factors involved in maintaining genomic integrity could be informative in understanding the cancer disease pathogenesis.

1.2 Breast cancer disease.

1.2.1 Incidence and mortality.

Breast cancer is the most common malignancy in women and a major cause of mortality and morbidity. Worldwide, it accounts for more than a fifth of all cancers, with an estimated recent incidence of more than a million new patients per year, resulting in almost 373,000 deaths (12, 13).

The incidence and mortality of breast cancer varies widely around the world, with North European countries, North America and Australia having the highest rates, and Asian and African countries the lowest (12, 14, 15) .In recent years, breast cancer incidence has been increasing in many countries, particularly in several Asian and African countries (15). Recent mortality rates have levelled off, or started to decline in many western countries, such as the UK, United States, Canada and Australia. Analysis of age-specific mortality rates shows that the change in mortality has occurred primarily in middle-age women and to a lesser extent, in older women. These trends are, at least in part, attributable to large-scale use of mammographic screening, early detection of breast cancer and, in particular, to the widespread use of adjuvant therapy (15-17).

In the UK, breast cancer continues to be the commonest cancer to affect women, with a recent annual incidence of nearly 44,000 new patients per year, accounting for almost 30 per cent of all new cancer cases in women, and with an estimated lifetime risk of one in nine. However, despite the increase in incidence, mortality rates have being falling steadily since 1990, probably due to a combination of factors, including earlier diagnosis and improved treatment. In the year 2001, there were 12,994 breast cancer deaths, compared to around 15,186 breast cancer deaths in 1992. It has been overtaken by lung cancer as the most common cause of death from malignancy in women (18).

In Scotland, breast cancer is by far the most frequent cancer among women, accounting for 27% of cancers in women and with approximately 3,533 cases in 2001. Over the last decade, a substantial improvement in breast cancer survival has been observed, with an obvious increase from 60% for patients diagnosed in 1977-1981 to 77% in 1997-2001. This improvement is likely to be due to a combination of new treatments, particularly

hormonal therapy, earlier diagnosis of cancers in women participating in the Scottish Breast Screening Programme, and better organization and delivery of care for patients. Around 1.2% of women are living with breast cancer and the prevalence of breast cancer is increasing due to improvements in prognosis and increasing incidence (Scottish Cancer Registry, ISD, NHS National Services Scotland; www.isdscotland.org.).

1.2.2 Aetiology and risk factors.

The search for specific breast cancer risk factors has been stimulated by the large difference in rates of breast cancer among countries worldwide, within countries over time, and by changes in rates among migrating populations. In general, the causes of breast cancer are not fully understood, but epidemiological researches have clearly identified important factors that influence breast cancer risk.

Several important genetic and environmental risk factors are associated with breast cancer. Approximately 5-10% of breast cancers have a genetic link; two thirds of these cases have mutations in the tumour suppressor genes BRCA1 and BRCA2. Mutations in these genes are associated with familial/hereditary breast and ovarian cancer, early age of onset, and cancer in both breasts (19). The main risk factor for sporadic breast cancer is age: the risk of developing breast cancer by the age of forty is 0.5%, by the age of eighty; it is 10% (20). The incidence of breast cancer increases rapidly with age during the reproductive years, being rare before the age of 25 and doubling about every 10 years until the menopause (about 50 years of age), when there is a slight levelling off before the incidence again rises, although at a reduced rate compared with the pre-menopausal years (21). The cumulative incidence of breast cancer among women in Europe and North America is about 2.7% by age 55, about 5% by age 65, and about 7.7% by age 75 (14).

Other risk factors include family history of breast cancer, prolonged exposure to oestrogen (onset of menstruation before age 12, menopause after age 50, first child born after the age of 30), alcohol abuse and obesity (Table 1.1).

Table 0.1:Risk factors for breast cancer, (21).

Factor	Relative risk	High risk group
Age	>10	Women aged over 50 years
Geographical location	5	Developed country
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first full pregnancy	3	First child in early 40s
Family history	≥ 2	Breast cancer in first degree relative when young (before age 50)
	4-6	Breast cancer in two first degree relatives (one before age 50)
Previous benign breast disease	4-5	Atypical hyperplasia
Cancer in other breast	> 4	
Socio-economic group	2	Group I and II
Diet	1.5	High intake of saturated fat
Body weight:		
Premenopausal	0.7	Body mass index > 35
postmenopausal	2	Body mass index > 35
Alcohol consumption	1.3	Excessive intake
Exposure to ionising radiation	3	Abnormal exposure in young females after age 10
Taking exogenous hormones:		
Oral contraceptives	1.24	Use during pregnancy
Hormone replacement therapy	1.35	Use for ≥ 10 years
Diethylstilbestrol	2	Use during pregnancy

1.2.3 Normal Breast Anatomy and Physiology.

1.2.3.1 Anatomy of the breast.

The female breasts are milk-producing glands, which vary enormously in their number and size, composed of lobules/glands, milk ducts, fat and connective tissue. Each breast contains 15-20 lobes, comprising lobules, which end in glands that produce milk in response to hormone signalling, arranged in a circular fashion. Ducts deliver milk to the nipple, thus connecting lobes, lobules and glands. Fatty tissue fills the spaces between the lobules and the ducts (Figure 1.1).Blood and lymph vessels form a network throughout each breast. The glandular tissue, with its various relative amounts of supporting connective tissue and fat, lies within a superficial fascia, in which fibrous processes (Cooper's ligaments) attach firmly to the deep and superficial layers of this fascia, and thereby to the overlying breast skin (22). The main bulk of the breast tissue is usually localized to its upper outer-quadrant.

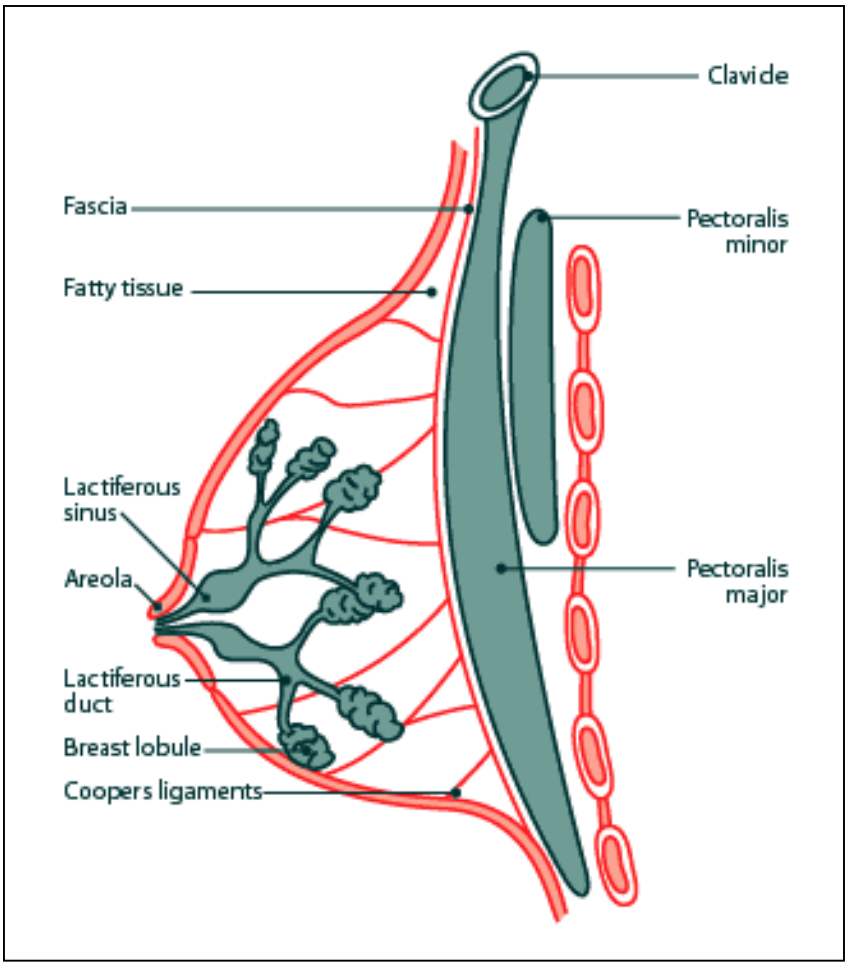

Figure 0.1: Anatomy of the breast:

Para- sagittal section of the breast, showing the normal anatomy of *the milk-producing gland.*

The adult female breast extends from the second rib above to the sixth rib below, and from the lateral border of the sternum medially to the mid-axillary line laterally; with its superiolateral extremity extending into the axilla as the axillary tail. On its deep aspect, about two-thirds of the breast lies on the pectoralis major. It overlaps the serratus anterior laterally and the upper part of rectus sheath inferiorly (22).

The nipple with its surrounding areola is usually situated at the level of the fourth intercostals space in nulliparous women, but this position tends to be variable with pendulous breasts. The nipple contains about 15-20 lactiferous ducts openings, and the areola contains numerous sweat and sebaceous glands(22).

The blood supply of the breast is a rich anastomotic network, which is derived from the internal thoracic artery (internal mammary), the intercostal artery, and the axillary artery

that branches into superior thoracic, pectoral branch of the acromiothoracic, lateral thoracic, and subscapular. These arteries are accompanied by their corresponding veins.

The lymphatics of the breast drain predominantly into the axillary lymph nodes, which receive nearly 75-85% of the breast drainage and into the internal thoracic (internal mammary) lymph nodes (Figure: 1.2). The axillary lymph nodes vary in number from 20-30 nodes, and often defined by clinicians and pathologists into three levels; as level I (nodes inferior to pectoralis minor), level II (nodes behind pectoralis minor) and level III (nodes above pectoralis minor). The apical nodes are also in continuity with the supraclavicular nodes and drain into the subclavian lymph trunk, which enters the great veins directly, or via thoracic duct or jugular trunk. The internal thoracic (internal mammary) lymph nodes are small, few (3-5 nodes) and lie along the internal thoracic vessels, deep to the plane of the costal cartilage (Figure: 1.2) (22).

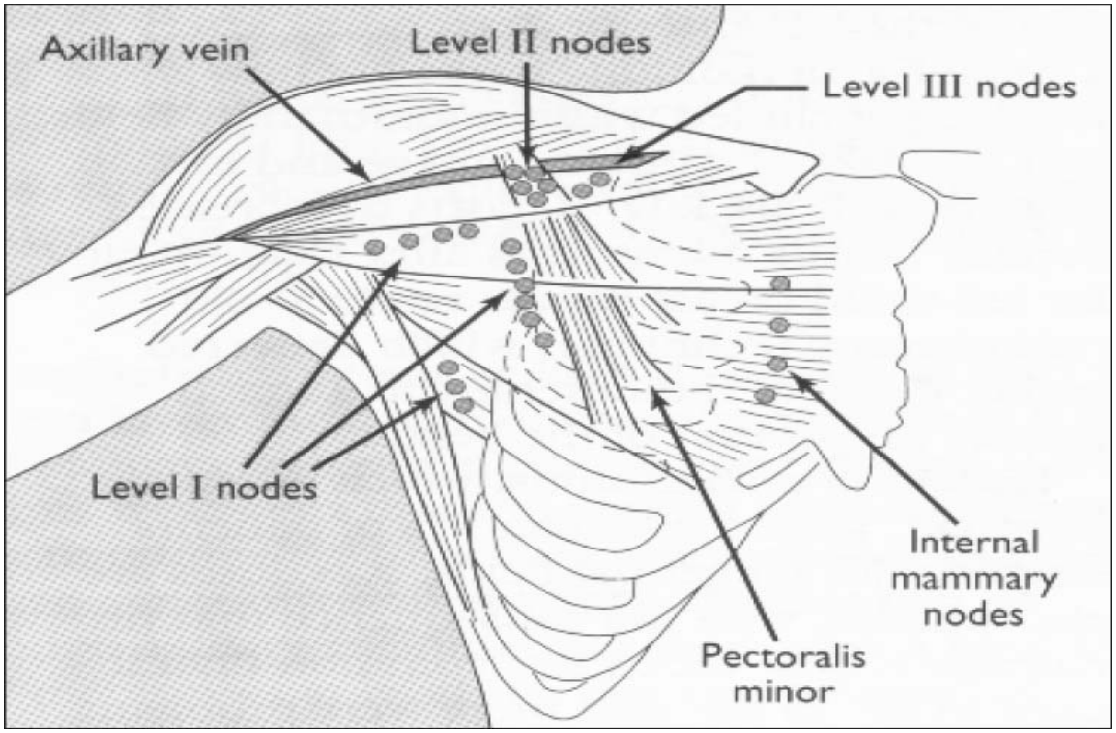

Figure 0.2. Surgical anatomy of the breast showing the lymphatic drainage of the breast.

1.2.3.2 Physiology of the breast.

The mammary gland undergoes three key phases of development and differentiation under the control of hormones and growth factors (23, 24): lobule development, between the ages of 10-25; glandular development, under the influence of menstrual hormones between the ages of about 13-45, and involution, or shrinkage of the milk ducts, from about age 35.

During the menstrual cycle, oestrogen stimulates milk duct growth; following ovulation, halfway through the cycle the hormone progesterone continues to stimulate milk gland formation. If pregnancy does not occur, the breasts return to normal size and the cycle begins again. During early (mammogenesis) and late (lactogenesis 1) phases of pregnancy, the final stages of development and differentiation occur. Mammogenesis is characterized by proliferation of the distal elements of the ductal tree, creating multiple alveoli (acini) of variable size and shape. Lactogenesis II occurs later and is characterized by differentiation of resting mammary cells into lactocytes, with the potential to secrete the unique fats, carbohydrates, and proteins characteristic of milk. The breast of nulliparous women contains more undifferentiated structures whereas the breast of parous women is comprised of more differentiated lobules (24). During lactation, mammary lobules become enlarged and milk is synthesised and released into the ductal system. Accumulation of milk in ducts has an inhibitory effect on the production of milk. This results in a series of involutional changes in the mammary gland, which reduces the volume of secretory epithelial cells and inhibits their secretory activity (24).

Hormone regulation is important, not only in the development and differentiation of the breast but also in the development of disorders, such as benign breast disease, and breast cancer.

1.2.4 Neoplasm of the breast.

1.2.4.1 Benign neoplasm.

The most common is a fibroadenoma, characterised by proliferation of the breast ducts and stroma. Others include the phyllodes tumour and benign intraduct papilloma.

1.2.4.2 Malignant neoplasm.

Breast cancers arise from the epithelial cells that line the terminal duct-lobular unit. The transition from a normal breast-epithelial cell into a cancer cell is assumed to proceed in a stepwise fashion in the multi-step phenomenon of breast carcinogenesis (Figure 1.3)(22, 25).

Breast cancer can be classified into pre-invasive (*in situ* carcinoma) and invasive cancer as follows:

1.2.4.2.1 In situ *carcinomas of the breast.*

In situ carcinoma is the proliferation of epithelial cells that have undergone malignant transformation but remain confined within the basement membrane at their site of origin within the terminal duct-lobular unit and draining duct. As there are no lymphatics or blood vessels in the epithelial layer, *in situ* carcinoma offers no risk of metastasis until malignant cells cross the basement membrane(22, 25).

Two types of *in situ* carcinoma of the breast have been described and comprise ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). Both arise from the terminal duct-lobular unit. However, there are significant clinical, morphological, and biological behavioural differences between the two types (Table 1.2,)

Traditionally, DCIS is classified on the basis of architectural and growth patterns into: comedo, cribriform, papillary, solid and micropapillary (Table 1.3). However, this classification has been superseded by another pathological classification that includes the nuclear grade of the tumour cells and the presence, or absence of necrosis (Van Nuys classification) (Figure: 1.4). Van Nuys classification stratifies the DCIS into three grades: low, intermediate and high. Higher grade (comedo) DCIS is most likely to develop into invasive cancer.

Table 0.2: Comparative features of ductal and lobular carcinoma in situ.

(26).

	DCIS	LCIS
Average age	Late 50s	Late 40s
Menopausal status	70% Postmenopausal	70% Premenopausal
Clinical signs	Breast mass	None
	Paget's disease	
	Nipple discharge	
Mammographic signs	Microcalcification	None
Risk of subsequent carcinoma	30% to 50% at 10 to 18 years	23% to 30% at 15 to 20 years
Site of subsequent invasive carcinoma		
-Same breast	99%	50% to 60%
-Other breast	1%	40% to 50%

Table 0.3 Types of carcinoma *In situ*.

Carcinoma In Situ can be categorised as ductal or Lobular.

Type of *Carcinoma In Situ*	Pathological Characteristics
Solid DCIS	Cancerous cells completely fill the affected breast ducts
Cribriform	Cells are very uniform in size and very regularly placed in relation to one another. Epithelial cells fill and expand the ducts and form defined glandular spaces separated by bridges of cells.
Papillary/Micropapillary	Commonly occurs in association with cribriform DCIS.
Comedo DCIS	Ducts are expanded due to proliferation of large pleomorphic cells. Characterised by mitotic figures and necrosis in the centre of the duct.
LCIS	Cells within breast lobes have undergone morphologic changes but are not cancerous. Lobules are expanded and filled by small, evenly spaced epithelial cells, which do not form ducts.

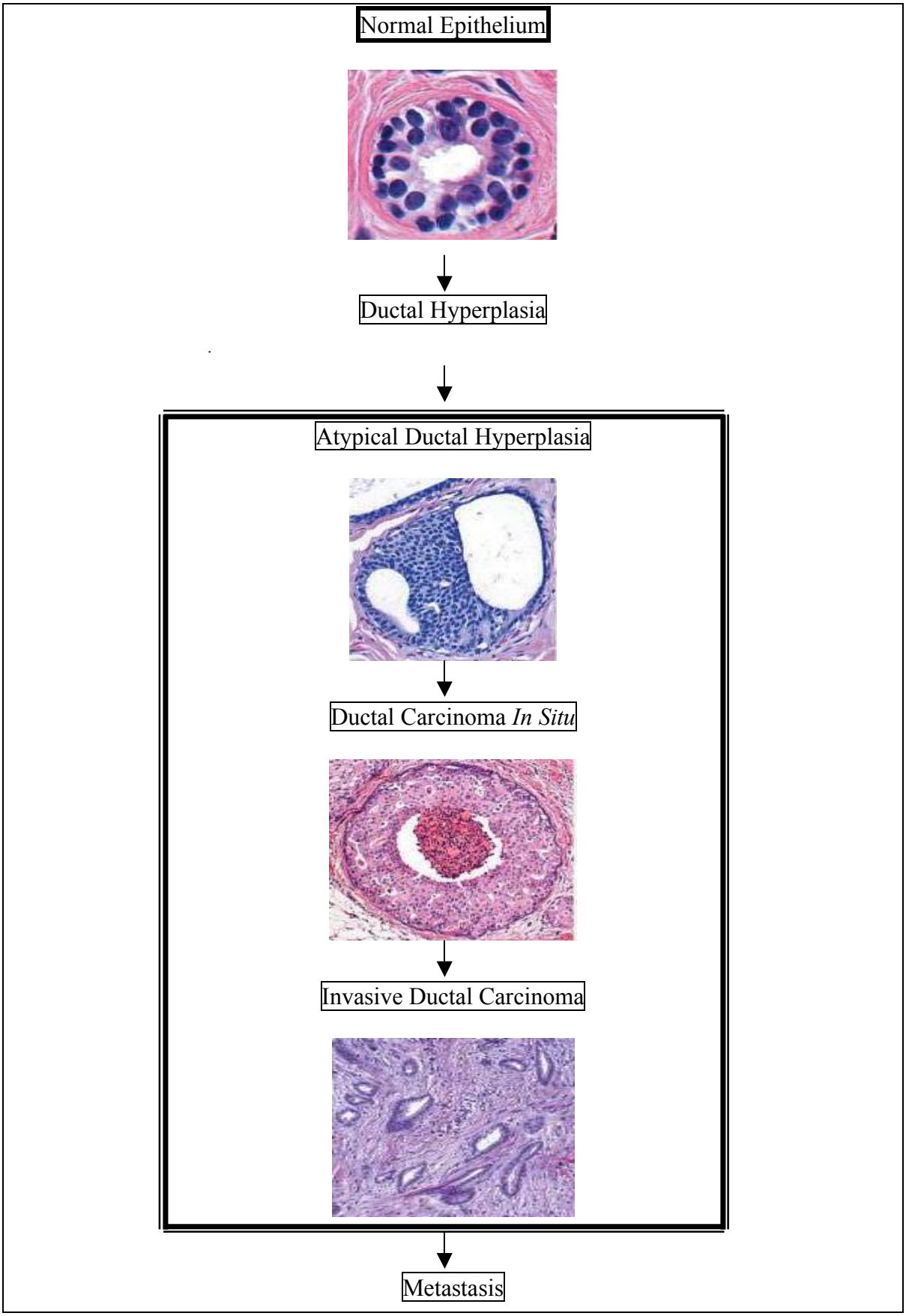

Figure 0.3: Multi-step breast carcinogenesis, (25, 27).

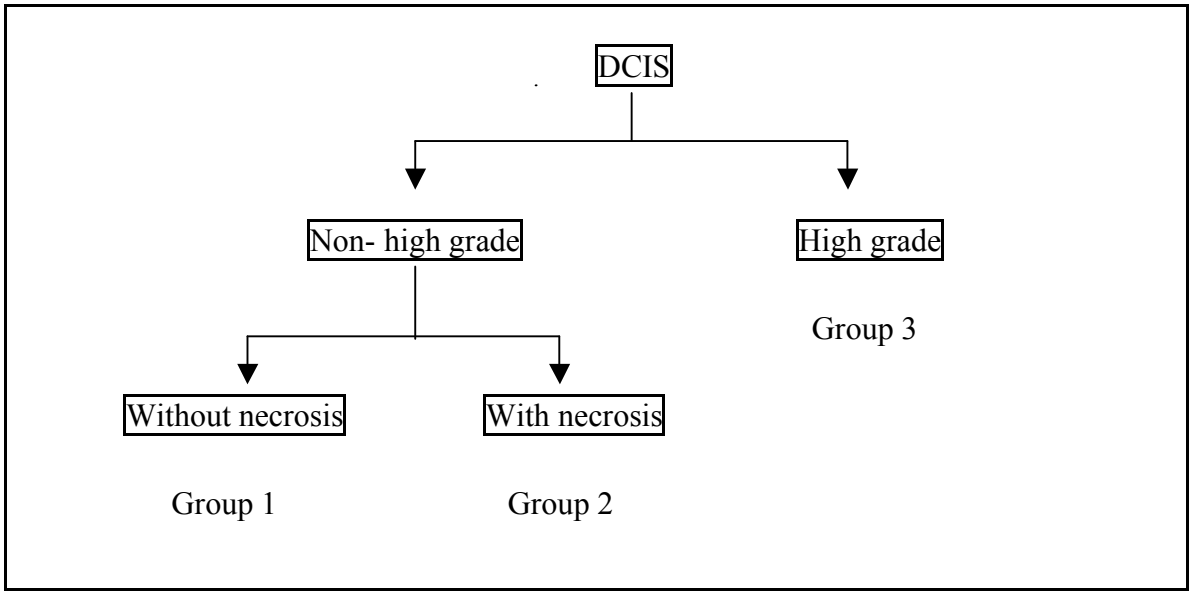

Figure 0.4: Van Nuys classification for ductal carcinoma *in situ* (DCIS) Classification, (28).

1.2.4.2.2 Invasive carcinomas of the breast.

Invasive breast carcinoma is defined as a malignant neoplasm of the mammary epithelial cells that have invaded beyond the native basement membrane at their site of origin, in the terminal duct-lobular unit, into the surrounding stroma. Invasive tumour cells may access lymphatic and blood vessels within the surrounding adjacent normal tissue, through which they may metastasise to both regional lymph nodes and distant sites (29)

Invasive breast cancers constitute a heterogeneous group of lesions that differ with regard to their morphological types, pathological features and biological potential. They are classified on the basis of the cytological and morphological features and growth pattern of the invasive tumour cells. rather than the structure of origin within the mammary duct system, as ductal carcinoma does not necessarily arise from the duct (22, 29). The main types are described below.

1.2.4.2.2.1 Invasive ductal carcinoma

Invasive ductal carcinoma is the most common histological type of invasive breast cancer, accounting for more than 50-70% of cases (22, 29, 30). It includes tumours with a range of histological appearance; from those with well-formed glands to those that have little or no evidence of specific differentiation, alternatively known as “carcinoma of no special type (NST)” and “carcinoma not otherwise specified (NOS)”, in which they are further categorised according to histological grade that takes into account the variation in

appearance (Mizrachi, 1999). Histologically, the tumour is often composed of cords and sheets of large pleiomorphic malignant epithelial cells that penetrate the stromal fibrous tissue haphazardly (Figure: 1.3).

1.2.4.2.2.2 Invasive lobular carcinoma.

Invasive lobular carcinoma is the second most frequent form, accounting for about 10-15% of all invasive breast cancer. It is more frequently multifocal and bilateral than IDC. Histologically, it is formed from moderately sized, regular malignant cells often arranged in linear cords that diffusely infiltrate within fine collagen bands, giving the "Indian file" appearance pattern (22, 30). ILC has a better prognosis than IDC, but is more likely to be bilateral.

1.2.4.2.2.3 Other invasive breast cancers.

1.2.4.2.2.3.1 Invasive tubular carcinoma.

Invasive tubular carcinoma is a special type of cancer that is typically associated with limited metastatic potential and excellent prognosis (31). It is uncommon in routine symptomatic practice.

1.2.4.2.2.3.2 Inflammatory breast cancer.

Inflammatory breast cancer is an aggressive type of breast cancer that develops rapidly, making the affected breast red, swollen and tender. This clinical manifestation is the result of cancer cells blocking the lymph ducts in the breast and prevent fluid from being drained from the tissue, resulting in inflammation of the breast.

1.2.4.2.2.3.3 Paget's disease of the nipple.

Paget's disease of the nipple is a superficial manifestation of an underlying breast carcinoma. It usually presents as an eczema-like condition of the nipple and the areola that does not response to conventional treatments.

Other special histological types of invasive breast carcinomas such as mucinous, medullary, papillary and invasive cribriform usually have a better prognosis (22, 31).

1.2.5 Spread of breast cancer.

Cancer treatment is challenging due to the ability of cancer disease to spread form the site of origin (primary cancer) to a different part of the body resulting in secondary cancer (metastasis).

In order to spread, some cells from the primary cancer must break away, travel to another part of the body and start proliferating. There are three main ways for a cancer to spread:

- Local invasion.
- Through the blood circulation (haematogenous spread).
- Through the lymphatic system.

1.2.6 Histological grading of breast cancer

Because of the histological diversity of breast cancer, histological grading is considered as an essential component of the pathological assessment of breast cancer. The grading system measures the degree of differentiation of a carcinoma by microscopic examination. Grading breast tumours is based on tubule formation (% of cancer cells composed of tubular structures), nuclear pleomorphism (changes in cell size and uniformity) and mitotic count (rate of cell division), each of which is scored from 1 to 3. Scores are totalled to provide a final score, ranging between 3 and 9, dividing tumours into three grades. Tumours with scores of 3-5 are considered Grade 1, well-differentiated tumours. Grade 2 tumours (score 6-7) are moderately differentiated, whilst tumours scoring 8-9 are Grade 3 (poorly-differentiated) (Table 1.4). Patients with grade 1 tumours have a better prognosis than those with grade 3 tumours (31-34).

Table 0.4: Histological grading system of invasive breast cancer.

Elston and Ellis modification of Bloom and Richardson grading system

Components of grading	Score
Tubules	
> 75% of tumour composed of tubules	1 point
10-75% of tumour composed of tubules	2 points
< 10% of tumour composed of tubules	3 points
Nuclear grade	
Nuclei small and uniform	1 point
Moderate variation in nuclear size and shape	2 points
Marked nuclear pleomorphism	3 points
Mitotic rate	
Dependent on microscopic field area	1-3 points
Histological grade	**Total points**
1 (Well differentiated)	**3-5**
2 (Moderately differentiated)	**6-7**
3 (Poorly differentiated)	**8-9**

1.2.7 Staging of breast cancer.

The prognosis of breast cancer is related to the stage of the disease at presentation. The staging depends on the size of the primary tumour (T) along with regional lymph nodes status (N) and the presence or absence of distant metastases. The T stages are numbered 1 to 4 and describe the size of the tumour. The N stages are numbered 0 to 3. They describe which lymph nodes are affected, if any. The M stages are M0 (no sign of cancer spread) and M1 (cancer has spread to another part of the body apart from the lymph nodes under the arm). This is known as TNM staging and is related to the clinical prognosis; patients with stage I tumours have a better prognosis than those patients with stage IV tumours (22, 30, 33).

Table 0.5: Breast cancer staging system.

Staging of a breast tumour depends on the tumour size, lymph node involvement and metastasis, and ranges from stage I to stage IV. T = Tumour Size: (T1 = 0-2 cm, T2 = 2-5cm, T3 = > 5cm, T4 = ulcerated or attached to skin or muscle), N = Node Status: (N0 = clear, or negative nodes, N1 = cancerous, or positive nodes), M = Metastasis: (M0 = no spread of tumour, M1 = tumour has spread).

Stage	Tumour Size	Lymph Node Involvement	Metastasis	TNM
I	Less than 2cm	No	No	T1, N0, M0
II	2-5cm	No, or in same side of breast (0-3 axillary lymph nodes involved)	no	T1, N1, M0 T2, (N0 or N1), M0 T3, N0, M0
III	More than 5cm	Yes, on same side of breast (≥ 4 axillary and/or internal mammary lymph node)	No	T3, N1, M0 T4, Any N, M0
IV	Any size	Any lymph nodes	Yes	T Any, N Any, M1

1.2.8 Prognostic and predictive factors in breast cancer.

The clinical course of breast cancer may vary from very slowly progressive to a course associated with rapid progression and metastatic spread. Therefore, assessment of certain prognostic and predictive 'markers' or 'factors' in the pathological examination of breast tumours, in order to predict disease outcome, is becoming increasingly important in understanding the natural history of breast cancer, planning treatment strategies and counselling patients. (35-37).

A prognostic factor is defined as any measurement, or feature, of the patient or tumour available at the time of diagnosis, or surgery, that is associated with outcomes, such as disease-free or overall survival, in the absence and independent of systemic therapy. As a result, is able to correlate with the natural history of the disease. Whereas, a predictive factor is any measurement, or feature, in which its presence, or absence, signals resistance or sensitivity, to a particular therapy independently of prognosis; it may, or may not, have prognostic value. Some factors, such as hormone receptors and Human Epidermal growth factor Receptor 2 (HER2/neu) over-expression, are both prognostic and predictive.(35-37).

Several established prognostic factors and a few predictive factors are used routinely in the clinical management of breast cancer. Through increased understanding of breast cancer biology, numerous other novel markers have been identified in recent years. These are in general either chronological; indicators of how long the cancer has been present, or

biological indicators of the metastatic potential behaviour of a tumour (35, 37) and are described below.

1.2.8.1 Age.

Breast cancer tends to be more aggressive with poor prognosis in younger patients than in older patients. Different studies have suggested that a young age of less than 35-40 years is, associated with the worst prognostic pattern, such as excess of high-grade tumour and axillary lymph-node metastasis, with the highest rates of necrosis, vascular invasion and proliferation and lower ER expression levels, and subsequently, a poorer survival rate. This poor outcome improves as age increases and is best in patients over 75 years of age. In addition, patient age is important for predicting response to chemotherapy and hormonal therapy, as menopausal status is an age-dependent factor (38-41).

1.2.8.2 Tumour size

Tumour size is a time-dependent prognostic factor that correlates directly with survival. The best measure of tumour size is maximum pathological size assessment, since radiological and clinical assessments may be inaccurate (38, 42). Patients with small tumours have a better prognosis, with a better long-term survival than those with larger tumours. The 20-year relapse-free survival rates for patients with tumours $\leq$ 10 mm in diameter have been reported to be around 88%. These rates fall to 72% and 59% for lesions measuring 11-30 mm and 31-50 mm in diameter, respectively. Nevertheless, 15-20% of tumours of $\leq$ 10 mm in size have nodal metastasis, compared with 40% for lesions of more than 15 mm (37, 38, 42-45).

1.2.8.3 Histological type/ grade.

The histopathological characteristics of the breast tumour have prognostic significance. Favourable prognosis of certain histological subtypes of invasive breast carcinoma, which based on architectural pattern of the tumour, is well established. Tubular, mucinous, papillary carcinomas, invasive cribriform, medullary, infiltrating lobular and tubulo-lobular types, together with rare tumours such as adenoid cystic carcinoma, adenomyoepithelioma and low-grade adenosquamous carcinoma have all been reported to have a better prognosis than carcinoma of no special type (ductal NST)(37, 38, 45).

The histological grading of breast cancer, (Table: 1.4), correlates strongly with prognosis. Patients with grade I breast cancer have the best survival rate with 85% 10-year survival, compare with less than 45% for those patients with grade III tumours. This histological grading potentially provides an overview of a number of molecular events that are reflected in histological morphology, which includes detail of cell morphology (nuclear pleomorphism), with a measurement of differentiation (tubule formation), and an assessment of proliferation (mitotic frequency) (34, 42, 44, 45)

1.2.8.4 Axillary lymph node status.

The presence, or absence, of axillary nodal metastases is the single most important prognostic factor for disease-free and overall survival in breast cancer. It is a time-dependent factor and has a direct correlation with survival from breast cancer. Clinical assessment of nodal status is unreliable; as palpable nodes may be enlarged because of benign reactive changes, or secondary to biopsy. Whilst nodes bearing tumour deposit may be impalpable. Thus, careful histological examination should be carried out for all excised axillary lymph nodes (42, 45, 46). A large number of studies with histologically confirmed lymph-node involvement have shown that, on average, 10-year survival is reduced from 75% for patients with no nodal involvement to 25-30% for those with metastatic disease in the locoregional nodes. Prognosis worsens with the greater the number of lymph nodes involved and metastasis to the higher axillary lymph nodes level, particularly those at the apex, which carries a worsened outcome (37, 38, 42, 43, 45, 46). Node positivity is reasonably taken as a marker of metastatic potential. However, this is only a qualitative, not a quantitative difference, as many patients with positive lymph nodes never develop distant metastases, while many with negative nodes do.

1.2.8.5 Lymphatic/ vascular invasion.

Lymphovascular invasion has been shown to be a significant prognostic factor in invasive breast cancer, particularly with respect to local recurrence and systemic relapse, and hence poorer overall survival. The presence of lymphovascular invasion, also, correlates closely with loco-regional lymph node involvement, tumour grade, and size (32, 37, 42, 44, 45).

1.2.8.6 Hormonal receptors.

In breast cancer, the levels of oestrogen (ER) and progesterone (PR) hormone receptor expression are considered prognostic and predictive factors (36, 37).

The oestrogen and progesterone receptors are steroid receptors located in the cell nucleus. Hormone is believed to diffuse into, or be transported to the nucleus where a steroid-receptor complex is formed with receptor dimerization. Some of the genes regulated by steroid receptors are involved in cell-growth control and currently, it is believed that these effects are the most relevant to oestrogen receptor, which influence the behaviour and treatment of breast cancer (45). As a prognostic factor, it has been reported that survival of women with ER positive cancers is longer. Again, with node negative breast cancer, ER status was a significant predictor of longer survival (38).

The value of these steroid receptors as a predictive factor for response to systemic endocrine therapy is well established. Approximately 30% of unselected patients with breast cancer will respond to endocrine therapy. However, with ER positive tumours, a response in about 50-60% of patients is seen compared with less than 10% in patients with ER-negative tumours (38, 45).

1.2.8.7 Human epidermal growth factor receptor-2.

The presence of human epidermal growth factor receptor-2 (HER-2 gene amplification and/or protein over-expression), usually detected by Immunohistochemistry (IHC) analysis and fluorescent in situ hybridisation (FISH), has been shown to have both prognostic and predictive value in human breast cancer (36, 47)

HER-2 status has attracted great interest as a potential predictor of endocrine and chemotherapeutic response, and more directly for the selective use of trastuzumab therapy; a humanized monoclonal antibody against HER-2 oncoprotein (35, 37, 47).

1.2.8.8 Tumour proliferation markers.

Tumour proliferation plays an important role in the clinical behaviour of breast cancer and many prognostic factors are directly, or indirectly, related to proliferation, such as cell-cycle regulators, growth factors and angiogenesis, respectively (Diest *et al*., 2004).

Cellular proliferation takes place through a defined process, in which several phases can be recognised. From the resting (G0) phase and after appropriate stimuli, cells join the active cycling population that enter the first gap (G1) phase. Both phases have a highly variable duration. In G1, the cell prepares for the synthesis (S) phase, in which DNA synthesis and doubling of the genome take place. The S phase is then followed by a period of apparent

inactivity, the second gap (G2) phase, in which the cell prepares for further separation of chromatids during the mitotic (M) phase. After the M phase, each daughter cell may enter G0 phase or move on to the G1 phase to repeat the cell cycle (48).

In invasive breast cancer, the prognostic values of various proliferation assays, including the S-phase fraction (SPF), thymidine labelling index (TLI), mitotic rate and Ki-67/ MIB1 index, have been shown to be associated with prognosis in the majority of studies. However, technical difficulties and lack of standardization in measurements, have limited their clinical usefulness. Nevertheless, mitosis counting and the Ki-67/MIB-1 proliferation index are considered to be the most practical methods to assess proliferation (37, 38, 48).

Mitosis counting, the oldest form of assessing proliferation, provides the most reproducible and independent prognostic value. It is the most well established component of the histological grading system in breast cancer (40, 48). Patients with tumour of increased mitotic index had a significant poor prognosis for overall and disease-free survival (44, 48).

Ki-67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle (G1, S, G2 and mitosis), but absent from the resting cells (G0), which makes it an excellent marker for assessing the growth fraction of a given tumour cell population, (38, 40, 44, 49, 50). The Ki-67 labelling index (Ki-67- positive tumour cells) has been shown to have strong correlations with other biological and histopathological markers of invasive breast cancer and also with the clinical course and outcome of the disease (38, 49, 50). The Ki-67 proliferation indices have been found to correlate directly with histological grade, tumour size, axillary lymph node status (51-53), vascular invasion, p53 and HER-2 overexpression, DNA and Thymidine labelling index score; and inversely with oestrogen and progesterone receptor status (Elston and Ellis, 1991; Querzoli *et al*., 1996; Morabito *et al*., 2003.).

1.2.8.9 Nottingham Prognostic Index (NPI).

The value of prognostic factors lies in a better quantifying of risk of recurrence and in defining low-risk patients, for whom adjuvant therapy is not indicated, and high-risk groups, who would most benefit from treatment. In 1982, a retrospective analysis was performed to investigate the prognostic capabilities of nine factors in primary, operable breast cancers: age, menopausal state, size, lymph node stage, tumour grade, cellular reaction, sinus histiocytosis, and oestrogen receptor expression (54). Three remained

significant, following multivariate analysis. These independent prognostic markers, lymph node stage, histological grade and tumour size were combined to form the Nottingham Prognostic Index (NPI) (Table 1.6). The NPI is calculated according to the following formula:

NPI = (0.2 x tumour size (cm)) + lymph node stage (1-3) + histological grade (1-3)

Currently, the Nottingham Prognostic Index (NPI) is the most practical integral measure available to assess individual patient's prognosis, and thereby, stratify appropriate adjuvant therapy for patients with invasive primary operable breast cancer (Table 1.6). In several independent prospective studies, the Nottingham Prognostic Index has been found to give valuable prognostic information, and due to its simplicity, it is suitable for routine clinical use (45, 54-58).

Table 0.6: Nottingham Prognostic Index (NPI).

NPI Score	Annual Mortality	15 years survival	Adjuvant Therapy
< 3.4	3%	80%	No systemic adjuvant treatment
3.4-5.4	7%	42%	Choice dependent on ER/PgR status, menopausal status & axillary disease
> 5.4	30%	13%	Aggressive adjuvant treatment: chemotherapy

Involved nodes	Tumour grade	Score /factor
0	I	1
1-3	II	2
>3	III	3

1.2.9 Management of breast cancer disease.

Breast cancer management is a rapidly evolving field. Optimal management with improved outcomes of patients with breast cancer have been achieved through a co-ordinated multimodality approach that require inputs from surgeons, radiologists, pathologists, oncologists, radiotherapist and psychologists with special interest in breast diseases, as well as general practitioners, breast-care nurses and the patient themself. Several studies have shown a better outcome with such multidisciplinary specialist treatment, which may relate to sufficient workloads and more frequent use of appropriate systemic therapy (59-61).

The purpose of the multidisciplinary approach is to design appropriate individualized treatment plans for all patients who require coordinated multi-specialty care. It lies in having a clear appreciation of the objectives of treatment and knowing its limitations and unwanted effects, in the context of the individual patient. The objectives that need to be considered include cure, local control, survival, and cosmetic, social and psychological consequences of the diagnosis and treatment of breast cancer.

1.2.10 Breast cancer treatment options.

1.2.10.1 Surgery.

In patients with early-stage breast cancer, surgery remains the first treatment modality to achieve local control, and in locally advanced breast cancer with fungating tumour, 'toilet mastectomy' may be required to control symptoms such as bleeding, discomfort, and pain.

The objective of surgery is to remove disease and identify extent of disease spread (staging). The surgical treatment of breast cancer has evolved into two categories; mastectomy (with or without breast reconstruction) and breast conserving surgery. Both should be combined with an axillary procedure.

1.2.10.1.1 Axillary management.

In breast cancer, axillary node status is crucial for staging, prognosis, and frequently directs the use of adjuvant systemic therapy in the management of early disease. It ranges from sampling; which should probably have four or more lymph nodes, to a level III clearance; which should yield an average of 20 or more lymph nodes.

Sentinel lymph node biopsy, a major development in the field of surgical oncology, has rapidly emerged within the last decade, as an alternative staging method to axillary lymph node dissection, to detect occult lymph-node metastases in patients with clinically node-negative invasive breast cancer (62, 63). It is based on the observation that specific areas of the breast drain, by way of efferent lymphatics, to a specific lymph node (sentinel lymph node), and then to other lymph nodes in the basin. As the sentinel node is the first node to receive the lymphatic flow from the primary tumour, it is assumed that if the sentinel node is correctly identified and is free of neoplastic cells, then the other axillary nodes are also negative. It is a minimally invasive technique that avoids the morbidity associated with formal axillary clearance, by excluding negative axillae from unnecessary dissection (63, 64).

The sentinel lymph node can be located by injecting a tracer material, such as blue dye, or radiolabelled particles, deep into or around the primary tumour (peritumoural), or superficially into the skin over the tumour site (subdermal, intradermal), or into the subareolar tissue. The blue dye allows the direct visualization of the lymphatic channels that lead to the marked lymph node in the axilla, whereas, the radioactive tracer facilitates the detection of the radioactive nodes prior to the surgical exposure of tissues by a hand-held gamma probe (63, 65-67). The identification rates have been demonstrated to be superior with a combination of both techniques, rather than either method alone (63, 68).

1.2.10.2 Radiotherapy.

Radiation therapy is an essential part of the management of breast cancer. It is indicated as a part of the primary treatment if breast-conserving surgery has been performed and in selected cases following mastectomy. It may be given to the breast, axilla, chest wall, and supra- and infra-clavicular fossa and internal mammary node chain. In patients with advanced disease, local radiotherapy may also be used as a palliative therapy to relieve symptoms, such as painful bone metastases (69). Radiation therapy uses high-energy radiation that kills cancer cells. A review of clinical trials involving radiotherapy over the last 40 years showed a significant reduction (~ two-thirds) in the number of patients relapsing following radiotherapy. Radiotherapy reduces breast-cancer related deaths, but increases deaths from other causes, in particular vascular-related deaths (70)

1.2.10.3 Chemotherapy.

The aim of adjuvant chemotherapy is to increase the cure rate after the primary loco-regional surgery. It is most frequently used in pre-menopausal patients, in patients with aggressive disease and with hormonal-receptor negative tumours (71).

Adjuvant chemotherapy is standard treatment for women with node-positive breast cancer. In a 10 year overview of approximately 100 clinical trials, for patients treated with adjuvant chemotherapy, recurrence risk was reduced by 22-37 % and risk of death by 14-27 % (72). Adjuvant poly-chemotherapy produced an improvement of about 7-11% in 10-year survival for women aged under 50 presenting with early breast cancer and by 2-3% for those aged 50-69 (73). Adjuvant chemotherapy is also beneficial to node-negative breast cancer patients. However, benefits must outweigh the risks of receiving cytotoxic agents. Chemotherapy is recommended for patients with moderately, to poorly differentiated, invasive tumours larger than 1cm or ER negative tumours. In the UK, patients' NPI scores guide chemotherapy use and the combination of epirubicin and Cyclophosphamide, Methotrexate and 5-Fluorouracil (CMF), is a common adjuvant practice in general.

Cyclophosphamide, an alkylating agent that is cell cycle specific, causes DNA strands to break apart, and impairs DNA replication. Methotrexate and 5-fluorouracil are cell cycle phase specific antimetabolites that inhibit the conversion of folic acid to its active form, folinic acid, preventing cell division and killing rapidly dividing cells (74). Anthracyclines, such as epirubicin, are antibiotics that induce apoptosis in tumour cells by disrupting the structure of the DNA. They do this by intercalating into the base pairs in the DNA minor grooves, and by causing free radical damage of the deoxyribose in the DNA (74).

In the adjuvant setting, currently used regimens include anthracycline-based chemotherapy, such as four courses of anthracycline and cyclophosphamide (AC), which have been shown to be equivalent to six cycles of classical cyclophosphamide, methotrexate and fluorouracil 5FU (CMF). The cyclophosphamide, epirubicin and fluorouracil (CEF), cyclophosphamide, anthracycline and fluorouracil (CAF) regimen and to some extent fluorouracil, epirubicin and cyclophosphamide (FEC) have yielded superior results. The CMF regimen is used less frequently but still valid for lower risk patients.

1.2.10.3.1 Taxanes chemotherapy.

In recent years, taxanes, such as paclitaxel and docetaxel, have been shown to be active cytotoxic antitumour agents (75). Taxanes act by shifting the dynamic equilibrium between tubulin and microtubules in the direction of microtubule assembly. Cells become blocked during the G2 and M cell cycle phases and cannot form a normal mitotic spindle and divide. Essentially, these microtubules are excessively stable and therefore dysfunctional (76).

Taxanes stabilize microtubules, enhancing the rate and extent of tubulin polymerization, and inhibit depolymerization. Microtubules have multiple functions in the eukaryotic cell, including the regulation of chromosome movement, cell morphology, hormone secretion, granule transport, and cellular motility, as well as the anchorage of receptors in the cell membrane (77, 78). Microtubules consist of protofilaments made of alpha- and beta-tubulin. Microtubule-assembly proteins (MAPs), such as Tau and MAP2, control the equilibrium between tubulin and microtubules. Microtubule formation is promoted by guanosine 5'-triphosphate (GTP), while depolymerization is promoted by high calcium levels and temperatures below $4^{o}C$ (77). Docetaxel binds to the beta subunit of the tubulin within the microtubule, promoting and stabilising microtubule polymerisation, while preventing physiological microtubule depolymerisation in the absence of GTP. This leads to a significant decrease in the number of free tubulin needed for the formation of microtubules which results in inhibition of mitotic cell division and cell proliferation leading to cell death by apoptosis (77, 78).

The taxanes (paclitaxel and docetaxel) are being incorporated into adjuvant regimens on the basis of their anti-tumour activity in advanced breast cancer and the absence of cross-resistance with doxorubicin (79). Many studies support the use of taxanes as adjuvant chemotherapy for women with early breast cancer and involved lymph nodes, independent of hormone-receptor status. The docetaxel-containing regimen (TAC) proved to be superior to CAF in randomized clinical trials and others trials have shown improvement in disease-free survival with or without significant improvement in overall survival in taxanes-treated patients (80-83).

1.2.10.4 Endocrine therapy.

The aim of endocrine therapy is to decrease the hormonal growth stimulation of hormone-sensitive breast cancer cells. It is indicated as adjuvant therapy or as treatment for advanced systemic disease and occasionally, as sole treatment in patients who are unfit or unwilling to have surgery (71, 84, 85).

An important determinant of patient treatment is the tumour hormone receptor status. Between 70-80% of breast cancers are oestrogen receptor (ER) positive and potentially oestrogen dependent. The presence of ER positive cells in breast tumours increases patients' chances of survival following endocrine therapy (86). The current options for hormonal manipulation include (71, 84, 85):

1. Therapies that reduce the levels of oestrogen through either:
    - Suppression or ablation of ovarian function through either:
        - Medically by luteinizing hormone releasing hormone analogues.
        - Surgical removal of the ovaries.
        - Radiation therapy.
    - Blocking the synthesis of oestrogen through the use of aromatase inhibitors
2. Therapies that antagonise the effects of oestrogen on the receptor by the use of
    - Selective Estrogen Receptor Modulators (SERMs) such as Tamoxifen.
    - Down-regulators of oestrogen receptor (Fulvestrant).

1.2.10.4.1 Oestrogen and oestrogen receptor targeted therapies.

1.2.10.4.1.1 Oestrogens.

Oestrogens are steroid hormones. There are three naturally occurring oestrogens . Oestrone (E1), 17β oestradiol (E2) and oestriol (E3), of which E2 is the main oestrogen from menarche to menopause (87). Oestrogens are synthesised in the ovary and testis, but also in peripheral tissues via the aromatisation of androgens. Ovarian synthesis of E2 dramatically

declines at menopause. The majority of breast cancers are diagnosed post-menopause, and non-ovarian sources of E2 are important in breast cancer development and progression. Following menopause, E1 becomes the predominant source of oestrogen.

In addition to its role in female reproduction, oestrogen is required for maintaining bone density and cholesterol levels. The relationship between oestrogen and breast cancer was demonstrated over 100 year ago with reports of a decrease in breast cancer growth after oopherectomy (surgical removal of the ovaries) reduced breast cancer growth (88). Oestrogen antagonists are now vital for the treatment of hormone dependent breast cancer.

The oestrogen effect is mediated through a receptor protein referred to as Oestrogen Receptors ER (89). There are two known isoforms of the oestrogen receptor, ERα and ERβ (90). ERα, a member of the steroid hormone nuclear receptor family, is a 65kDA ligand-activated transcription factor. Oestrogen promotes release of growth factors by ERα positive breast cells, which stimulate proliferation, in a paracrine manner, of neighbouring ERα negative cells (91). 15-20% of normal breast cells express ERα, and these cells are generally non-dividing. Patients diagnosed with ERα positive tumours have a better prognosis than patients with ERα negative breast cancers (38, 45), and ERα tumours are more responsive to hormonal therapy than ERα negative tumours (92, 93).

1.2.10.4.1.2 Tamoxifen.

For the last 20 years, Tamoxifen treatment has been the gold standard for ERα positive breast cancer patients. It is currently one of the most effective but least toxic drug therapies. Tamoxifen, an oestrogen antagonist, it is a non-steroidal drug that acts as a selective oestrogen receptor modulator (SERM). It competitively inhibits the interaction of E2 with the ERα.

Tamoxifen inhibits only AF-2 functions; it does not interfere with AF-1 activation. Consequently, Tamoxifen may function as both an ERα antagonist and agonist as result of its interaction with different functional domains of ERα. This is responsible for the side effect of Tamoxifen therapy as it functions as an oestrogen agonist in the uterus, ERα activity is more reliant on AF-1 activation; therefore Tamoxifen functions as an agonist. Thus stimulates endometrial proliferation, which increases the risk of endometrial cancer (~2.5 fold) (94).

1.2.10.4.1.3 Fulvestrant.

To combat the agonistic effects of Tamoxifen, "pure antioestrogens" that do not demonstrate agonistic behaviour were developed. Fulvestrant (Faslodex), has no agonistic effects and has a 100 fold greater binding affinity for ERα than Tamoxifen, and is approved for treatment of ERα positive, metastatic, post-menopausal breast cancers. Fulvestrant binds ERα, prevents DNA binding and promotes ERα degradation, markedly reducing cellular receptor levels (95). Tamoxifen resistant cell lines respond to Fulvestrant (96).

1.2.10.5 Biological therapy in breast cancer.

Trastuzumab (Herceptin®) is a humanized monoclonal antibodies with anti-tumour activity targeted against the epidermal growth factor family oncogene (Her-2/*neu*). It is a novel therapeutic option for patients with aggressive forms of advanced metastatic Her-2/ *neu*-positive breast cancer (Ross *et al*., 2003; Vogel and Franco, 2003).

It suppresses tumour growth when used as a single agent, or in combination with other chemotherapeutic agents. Single-agent trastuzumab therapy produces objective benefits in 15-20% of patients with Her-2 positive tumours. Trastuzumab, in combination with cytotoxic chemotherapy, either anthracycline plus cyclophosphamide, or taxanes, has been shown to improve overall survival, with a higher rate of objective response and a longer duration of response, as well as, increased time to disease progression and time to treatment failure, in synergistic, rather than an additive, manner. Ongoing trials of trastuzumab in combination with various chemotherapy agents are encouraging and showing significant clinical activity over chemotherapeutic regimens alone (97-99).

Trastuzumab therapy is generally well tolerated. Mild side-effects may include fever and chills; which are generally seen with first infusion dose, in addition to, diarrhoea, nausea, headache, rash and rhinitis. Other severe adverse events include cardiac toxicity, especially occurring when used in combination with anthracycline-based chemotherapy regimens. In the absence of pre-existing cardiac disorders, or prior anthracycline therapy, intrinsic cardiotoxicity with single-agent trastuzumab appears to be rare(97-99).

1.3 Biological ageing and cancer.

1.3.1 Ageing and replicative senescence.

In man, replicative senescence (RS) occurs when a somatic cell reaches the end of it replicative potential. Typically, this is after a finite number of cell divisions and is termed the Hayflick limit (100, 101).

During this active period (proliferation), cells accumulate damage and it is well known that ageing is a consequence of this permanent damage accumulation to molecules and cells. In wider view, ageing is a result of accumulation of this damage to the cells, organs and tissues that compose the organism. This damage is caused by various intrinsic and extrinsic biological and biochemical stresses. Different maintenance mechanisms, which are designated to control pathways that influence the rate of biological and biochemical stresses, and to counterbalance the accumulation of resultant damage, are essential for the preservation of health and longevity. Two different concepts of age have been determined: chronological age and biological age. Chronological age is dictated by time elapsed since birth, whereas the biological age is dictated by how much damage has accumulated over the chronological age and how effective maintenance mechanisms were in counterbalancing this damage. Chronological age and biological age do not always equate.

There are striking differences in the life spans and onset of ageing between species. On average mice live 2.5 years, monkeys 30 years and humans about 80 years. Organismal ageing is manifested by a progressive decline in vitality over time leading to death. At the organ level, ageing manifests as declining organ mass, cellular function, and integrity with time elapsed since birth. It reflects the lifestyle of the organism, life history and the cumulative burden of oxidative insult at the molecular level (102).

The difference in the organism's content of post mitotic and/or self-renewable cells might be one of the essential factors determining life span in different organisms. In postmitotic organisms, the accumulation of damage that underlies ageing will lead to a permanent loss of cells which cannot be compensated for. However, organisms with self-renewable tissues can replace damaged and lost cells, thus increasing their possibilities for maintenance, repair and longer lifespan. However, the capacity for cellular self-renewal also brings an enormous danger not experienced by post-mitotic organisms, namely DNA damage accumulation, leading to genomic instability and resulting cancers (103). To protect against cancer risk, different tumour suppressor mechanisms have evolved, which can be

regarded as caretaker (protecting the genome against mutation) and gatekeeper (inducing cell death, or cell cycle arrest of potentially carcinogenic cells) functions. Incremental telomere erosion, which accompanies somatic cell division, acts as counting mechanism for determining when a cell should enter growth arrest and has been considered as an anti-neoplastic mechanism.

1.3.1.1 Telomeres and ageing.

Telomeres are nucleoprotein complexes found at the end of eukaryotic chromosomes. In mammals, telomeres consist of a dynamic complex of proteins bound to a multiple TTAGGG nucleotide repeats (104). Mammalian chromosomes undergo attrition with each cellular division. Telomere length is, hence, an indicator of the replicative history and replicative potential of these cells. This feature of telomere biology is at the core of the concept that, at the cellular level, telomeres serve as a mitotic clock. In human beings, telomere length is heritable, relatively short, highly variable and inversely related with age, especially in replicating somatic cells. Because of the long lifespan of humans and their short telomeres, attrition in telomere length may be a major determinant of human ageing, not only at the cellular level, but also at the organ and perhaps the systemic levels. Not only changes in telomere length, but also changes in its components can trigger senescence or apoptosis. These changes can result in the loss of Telomere Positional Effect (TPE) and the expression of sub-telomeric genes whose expression may contribute to the senescent phenotype. Since telomeres act as a sink for DNA repair proteins such as Ku, damage signalling component such as sirtuins and damage checkpoint regulators, such as taxia telangiectasia mutated (ATM), the re-localization degree of proteins from the telomere to the injury site, may act as an indicator of damage level (2). This leads to induction of apoptosis, growth arrest, or repair. Cells may therefore senesce and exhibit growth arrest not solely by replicative senescence and the shortening of telomere lengths but also by alterations in telomere status.

Senescence at the cellular and physiological levels is thought to contribute to both disease and ageing (2, 105). Moreover, it is also believed that in order for mammalian cells to undergo malignant transformation, senescence associated growth arrest must be overcome. Therefore, telomere erosion has been considered as an anti-neoplastic mechanism that minimizes the proliferation of mutant cells that may give rise to tumours in long-lived mammals such as man. The maintenance of telomeric length would therefore be important for cells to overcome this senescent arrest. The finding that telomere lengths are actively maintained in many tumours, by compensatory telomere maintenance activity (via

telomerase, or alternative lengthening of telomere (ALT), fits with this hypothesis (106). Consistent with the concept that telomere status rather than only telomere length, can trigger senescence, factors that are involved in maintaining telomeric stability, such as sirtuins, might also be involved in tumourigenesis.

1.3.1.2 Age and disease.

The central tenet of geriatrics is that ageing is not a disease. This is true when the chronological age equals the biological age. The functional decline that accompanies normal ageing has been well characterized (107), but under normal circumstances does not account for symptoms. For example, that kidney function declines with age is well recognized. In fact, this decline in kidney function has proven to be a useful biologic marker of ageing in humans (108). However, clinical consequences of this change in renal function—in the absence of a disease, or the exposure to an exogenous nephrotoxic agent—do not occur, except when the organ is transplanted (109).

Therefore, biological ageing (accelerated) that does not equate to chronological ageing, regardless of the cause that could be either internal (genetic), or external (environmental) factors, or a combination of both, is responsible for overt disease, not the normal ageing process. Ageing is not a disease process, but the changes in physiological reserves that accompany ageing may make an individual susceptible to disease. For example, the immune response declines with age, but is not of sufficient magnitude or duration to account for the increased incidence of cancer in old people (110).

Therefore, a different approach to correlating biological ageing and diseases was necessary. Utilizing a biological ageing marker (telomere length) proved to be useful and informative in heart disease (111), inflammation (112), physical health, cognitive ageing (113) and scleroderma (114). Similarly a biological marker (telomere length) was informative in chronic kidney disease (115). Furthermore, the biological age of the donated kidney, as indicated by a range of biological markers (CDKN2A(p16), CDKN1A(P21), SIRT2, XRCC5 and HPOT), is associated with kidney function after transplantation (109).

1.3.1.3 Age and cancer.

The majority of people diagnosed with cancer are over 65; 60% of newly diagnosed cancers are found in people over the age of 65. Overall, people in this age group are 10 times more likely to get cancer, and 15 times more likely to die from the disease, than are people under the age of 65. Therefore, cancer is considered as a disease of ageing because it develops primarily in older adults, Furthermore, ageing is considered the most potent of all carcinogens (116). In humans, the incidence of cancer rises exponentially in the final decades of life, beginning at about the mid-point of the maximum lifespan (116, 117). This dramatic age-dependent escalation in cancer risk is fuelled largely by a marked increase in epithelial cancers, as opposed to cancers of mesenchymal, or haematopoietic origin. It is generally believed that the cancer-prone phenotype of older humans might reflect the combined effects of cumulative mutational load, increased epigenetic gene silencing, telomere dysfunction and altered stromal milieu (116).

1.3.1.3.1 Age and tumour suppressors.

Cancer and ageing are also linked, because the molecules that control tumourigenesis, e.g. tumour suppressors, also regulate ageing and lifespan. It turns out that cellular senescence and apoptosis, induced by increased expression of tumour suppressors that have evolved to protect complex organisms from malignant tumours, may also contribute to development of the cancer-prone phenotype in later stages of life. In general, tumour suppressors can be divided into caretakers and gatekeepers (103, 118). Caretakers protect the genome from damage, or mutation, to ensure genomic stability, but usually do not directly stimulate cell proliferation. Candidates for caretaker tumour suppressors are BRCA1 and BRCA2. In contrast to caretakers, p53 and Rb functions as gatekeepers to eradicate potential cancer cells through apoptosis, or to suppress their proliferation through cellular senescence. Mutations inactivating any kind of tumour suppressor will increase the risk of developing cancer. Therefore, all tumour suppressors, in theory, should directly promote the longevity of the organism by preventing the development of malignant tumours. However, the relationship is more complex and tumour-suppressing mechanisms turn out to actually accelerate ageing and limit longevity according to an evolutionary hypothesis known as antagonistic pleiotropy (119, 120).

Tumour-suppressor mechanisms, particularly the gatekeeper mechanisms of apoptosis and cellular senescence, suppress the development of cancer, but promote the development of specific ageing phenotypes. Firstly, both apoptosis and cellular senescence contribute to

the depletion of the renewal capacity of tissues by exhausting the supply of progenitors or stem cells. Secondly, senescent cells, resembling carcinoma-associated (activated) fibroblasts, secrete degradative enzymes, cytokines and growth factors (121) and contribute to ageing, by actively disrupting the integrity, function and/or homeostasis of tissues as they accumulate.

The antagonistic pleiotropy hypothesis proposes that traits benefiting young organisms can have unselected deleterious effects later in life. Although they protect organisms from cancer early in life, they may promote ageing phenotypes, including late life cancer, in older organisms. Moreover, when caretaker mechanisms fail, the ageing phenotypes that develop might derive not only from the loss of genomic integrity, but also from the apoptosis and/or cellular senescence that can occur in response to the accumulated damage. So, the caretaker and gatekeeper tumour suppression mechanisms can interact. In addition to tumour suppressors, the over-expression of oncogenes is also linked to cellular senescence through oncogenic stresses that activate tumour suppressors, further complicating the already complex relationship between ageing and tumourigenesis. Overall, ageing and tumourigenesis appear not to be separable at the molecular level and molecules that regulate one are likely also to regulate the other. Thus, ageing and cancer may be considered as distinct phenotypes arising from changes affecting a similar set of molecules. For example, senescent cells are arrested mainly in the G1 phase and mutations involving the G1 checkpoint are extremely common in human cancer.

1.3.2 Epigenetics and cancer.

Much effort has been invested in identifying genetic mutations in cancer. This approach has proved to be successful in inherited cancer syndromes. However, mutations early in the genesis of common cancers have also been identified and these are likely to be associated with tumour initiation. In contrast, few specific genetic mutations have been linked to tumour progression, leading Feinberg to suggest that epigenetic changes may be involved (122). Epigenetic changes occur without a change in the DNA sequence and they can be induced by various factors. Thus it is possible, for example, that a DNA mutation leads to cellular transformation, but induced changes in the epigenome of the transformed cell enhances the probability that it will be capable of metastasising. In this scenario, a genetic mutation initiates the cancer, but epigenetic change promotes its progression.

It is possible that epigenetic change may lead directly to cancer initiation. Alternatively, changes already induced within the epigenome may 'prime' cells in such a way as to

promote cellular transformation, upon a subsequent DNA mutagenic event. In this case, the epigenetic component of the cancer initiation is intricately entwined with the genetic component (122). The involvement of epigenetic change in cancer initiation does not exclude it also having a role in cancer progression.

A genetic alteration in the gene encoding epigenetic enzymes (e.g. histone acetyltransferase and deacetylases) may lead to changes within the epigenome. If, for example, these changes cause the activation of an oncogene, then cancer may arise. In addition, mutations in genes that code for proteins that recognize and bind to epigenetic marks (e.g. methyl binding domain proteins and bromo/chromo domain proteins which, bind to methylated DNA and acetylated/methylated histones respectively) could be as important in cancer as mutations in the enzymes themselves. Although these are genetic events that lead to cancer, an alteration in the epigenome most likely also plays a part. However, it should be noted that many of the histone-modifying enzymes also modify non-histone proteins, thus making a direct link between enzyme deregulation, changes in the epigenome and cancer extremely difficult to unambiguously demonstrate. In this context it is clear that the deacetylase actions of sirtuins implicate them in ageing and cancer processes through epigenetic effects.

1.4 Sirtuins

1.4.1 Silent information Regulator2 (SIR2).

The term silent information regulator 2 (SIR2) was designated to describe a gene and its encoded protein required for the transcriptional repression of the silent mating type loci, HML (homothallic left) and HMR (homothallic right), in the budding yeast *Saccharomyces cerevisiae*. Silencing is a regional inactivation of transcription at a specific chromosomal domain and requires a set of non-histone chromatin-associated components, including the Sir2 protein and its homologues. Silent chromatin has a specialised, packaged, condensed and latent structure that prevents transcription, replication and other DNA modifications, such as methylation and endonucleation (8, 123). The SIR2 gene belongs to a big family of histone deacetylases. The finding of *SIR2* homologues in yeast and shortly thereafter, in organisms ranging from bacteria to plants and mammals, demonstrated that SIR2 is a member of a large and ancient family of genes. In mammals seven Sir2- like proteins, termed sirtuins has been characterised.

1.4.1.1 The histone deacetylase family.

Transcription in eukaryotic cells is influenced by the manner in which DNA is packaged (124). In resting cells, DNA is tightly compacted to prevent accessibility of transcription factors. DNA is packaged into chromatin, a highly organized and dynamic protein ± DNA complex. The fact that acetylation is a key component in the regulation of gene expression has stimulated the study of histone deacetylases (HDACs), in relation to the aberrant gene expression often observed in cancer.

Acetylation is controlled by two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs deliver acetyl groups to the lysine residues in the amino terminal tail of core histones, neutralizing the positive charge, and resulting in the unwinding of chromatin. This modification provides docking sites for regulatory proteins to activate transcription. HDACs, on the other hand, induce transcriptional repression and gene silencing by catalyzing the removal of the acetyl groups from core histones.

In humans, eighteen histone deacetylases (HDACs) have been identified is divided into four classes based on their homology to yeast proteins. In general there are two families of deacetylases identified in eukaryotes, the classical histone deacetylases family (class I, II,

IV), and the recently discovered sirtuins family (Class III). Each of these two families is divided into subclasses as follows:

- The classical HDAC family is divided into three classes based on their homology to yeast proteins. The class I HDACs, namely 1, 2, 3 and 8, are ubiquitously expressed nuclear proteins and most closely related to the yeast (*Saccharomyces cerevisiae*) transcriptional regulator RPD3. Class II HDACs (4, 5, 6, 7, 9 and 10) share domains with similarity to another deacetylase found in yeast HDAC1, and can be found in both the nucleus and the cytoplasm. HDAC11 is remotely similar to class I and II HDACs but the sequence identity is too weak to be placed in either class. It is thus designated as the sole member of class IV. Currently, it is thought that HDACs of class I are expressed in most cell types, whereas the expression pattern of class II HDACs is more restricted, suggesting that they might be involved in cellular differentiation and developmental processes (125, 126). All members of class I, II and IV HDACs are sensitive to the inhibition by trichostatin A (TSA).

- Sirtuins family, class III histone deacetylases, is divided into five classes (I, II, III, IV, U) SIRT1, SIRT2, and SIRT3 belong to class I, which groups all yeast sirtuins and also at least one of the Sir2-related proteins in most eukaryotes. SIRT4 belongs to Class II, which also includes sirtuins from bacteria, insects, nematodes, mould fungus and protozoans. SIRT5 belongs to class III that is widely distributed in all prokaryotes either bacteria or archaea. Finally, SIRT6 and SIRT7 belong to class IV, and unlike Class III, sirtuins of this class are not present in prokaryotes, but are broadly distributed in metazoans, plants and vertebrates. Additional class (U) has been assigned for bacterial Sir2 homologues that have undifferentiated motifs that are intermediate between classes II and III, and classes I and IV. Sirtuins from Class II, Class III and Class U (undetermined) seem to have evolved earlier than the other classes. Thus, SIRT4 and SIRT5 may be the most ancient mammalian sirtuins. Class I and Class IV sirtuins, which group SIRT1, SIRT2 and SIRT3 and SIRT6 and SIRT7 respectively, are only present in eukaryotes. Thus sirtuins from Class II and Class III together with Class U seem to be the ones that appeared earliest in evolution (127, 128).

This classification has been decided upon phylogenetic analysis of 60 core domains from different eukaryotes and prokaryotes. All SIR2-like proteins have a common core domain which contain a series of conserved sequence motifs, comprising 275 amino acids, in

organisms ranging from bacteria to man (129) (Figure 1.5). Sometimes an additional N-terminal or C terminal sequence is present (127). All have an NAD^+-dependent catalytic core domain that may act preferentially as a mono-ADP-ribosyl transferase (ART) and/or NAD^+-dependent deacetylase (DAC). Additional N-terminal and/or C-terminal sequences, of variable length, may flank this core domain. The seven sirtuins show different cellular localization.

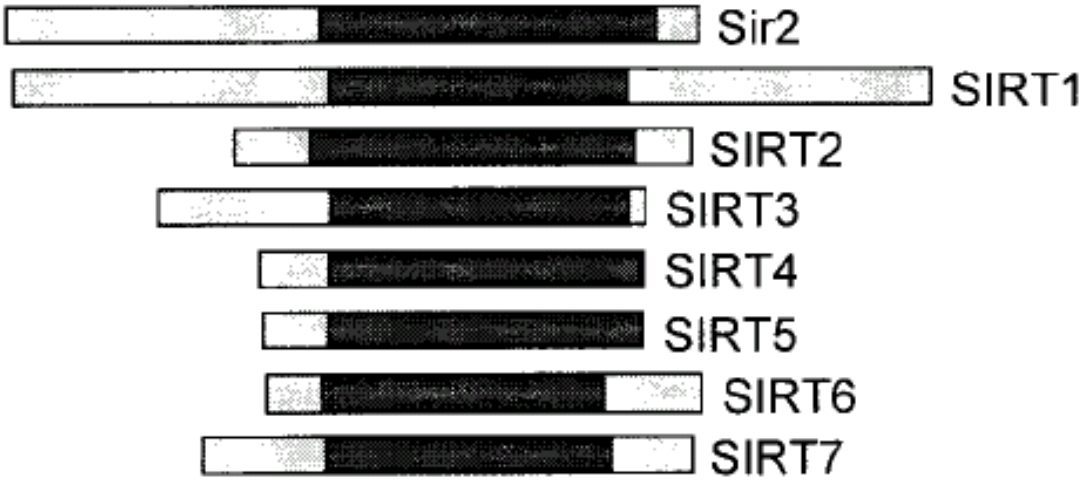

Figure 0.5: The position of the conserved core domain (dark shading) of Sir2 from *S. cerevisiae* and the seven human sirtuins. Modified from Frye, 2000.

1.4.2 Functions of the Silent information regulators (Sir).

Sir proteins are involved in diverse processes ranging from regulation of gene silencing at telomeres and mating type loci, DNA repair (double-strand break repair), structure and function of the nucleolus, suppression of ribosomal RNA (rDNA) recombination, regulation of the mitotic cell cycle, meiosis and ageing (8, 9, 130). *SIR2* in *Saccharomyces cerevisiae* is the best characterized and the best-studied member of this family. The enzymatic activity, structure and essential functions are described below:

1.4.2.1 Sir2 Structure.

The structure study of Sir2 showed that the conserved core domains of the protein form two lobes. The large domain contains a Rossmann fold, and the small domain contains a helical loop, a three-stranded zinc ribbon motif and a flexible loop. A conserved large groove is inter-positioned between these two domains. The large groove contains, between

the Rossmann fold and the helical loop, the NAD binding site, accessibility to which is controlled by a flap formed by the flexible loop (131, 132). It also contains conserved hydrophobic and a hydrophilic lining motifs. The hydrophobic motif has been proposed to be the lysine deacetylation site. Moreover, the hydrophilic motif, containing poorly conserved polar residues, has been implicated in control of the enzyme substrate specificity (133) (Figure 1.6).

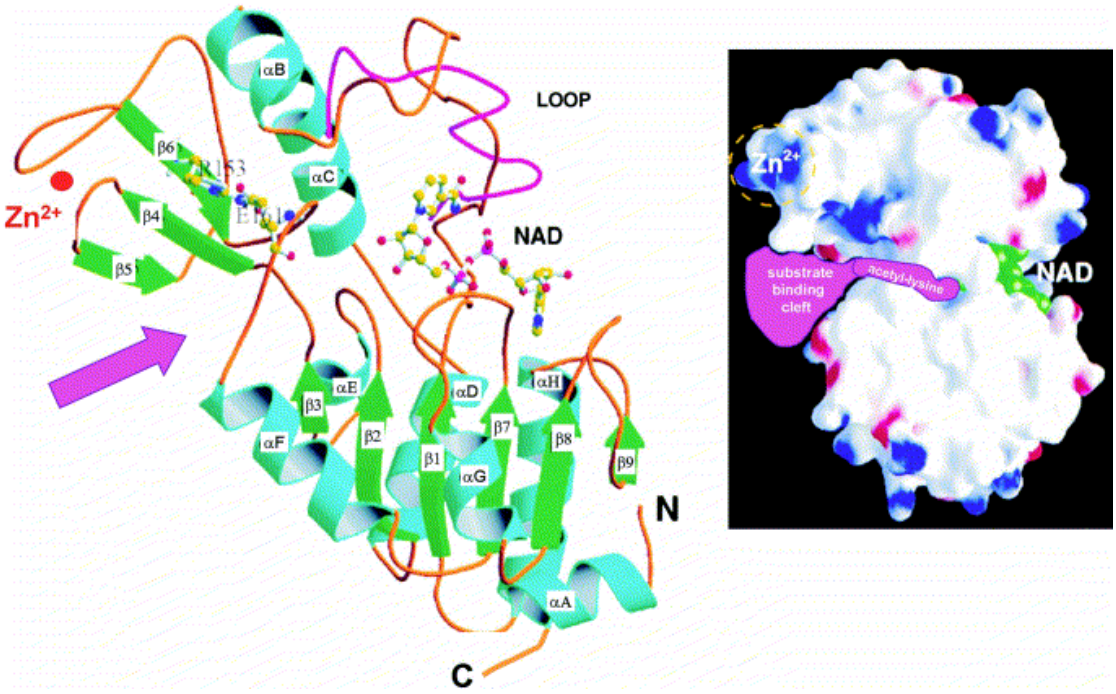

Figure 0.6: Crystal structure of SIR2.

The arrow indicates the groove between the two domains, which contains the substrate binding site and the NAD binding pocket. The flexible loop (flap) above the DNA-binding pocket is also shown. Adapted from Gasser and Cockell, 2001.

1.4.2.2 Enzymatic activity.

The Sir2 proteins are class III histone deacetylases (127) and the observation that Sir2 over-expression resulted in global hypoacetylation of histone gave rise to the rational thought that Sir2 was responsible for deacetylation of nucleosome-associated histones (134). Consistently, further researches suggested that the enzymatic activities were important for the role of Sir2 in telomeric, HM, and rDNA repression (133). Even though initial studies showed that Sir2 protein functions as a NAD (nicotinamide adenine dinucleotide)-dependent ADP (adenosine diphosphate) ribosyl transferase (7), further research has provided evidence that Sir2 functions primarily as a deacetylase, which uses NAD as a cofactor (135). Consistent with Braunstein's speculation, deacetylation has been confirmed as the main function of Sir2, and the previously mentioned ADP-

ribosyltransferase activity has been described as a 'low-efficiency side reaction' (135). The Sir2-dependent deacetylation reaction is tightly coupled with NAD cleavage and the formation of a previously unidentified product, 2-O-acetyl-ADP-ribose (OAAR) (Figure 1.7). The Sir2 histone deacetylase activity has been shown by two different groups to be absolutely dependent on NAD^+ (136, 137). OAAR is now understood to be a tightly coupled by-product of deacetylation reaction catalysed by the Sir2. Furthermore, the production of the OAAR metabolite itself has an important function in delaying/blocking oocyte maturation as well as embryo cell division in blastomeres (138, 139).

Unlike the other deacetylase classes, sirtuins do not have a zinc catalysed function and are not inhibited by trichostatin.

Initially, it has been speculated that Sir2 may have another substrate, other than histones, as it present in almost all organisms, including bacteria, which have no histone proteins. This was supported by identification of extranuclear locations for Sir2 (140, 141). This has been supported by subsequent observations demonstrating that Sir2 orthologues is able to deacetylate a number of substrates including p53, Ku70, and Foxo transcription factors (142-146) in man and the Taf168 subunit of the Tata-box binding protein-containing factor in mice (147).

1.4.2.3 Nicotinamide and Sir2 enzymatic activity.

Nicotinamide, a vitamin B3 precursor, is a product of the Sir2 deacetylase reaction and potent non-competitive inhibitor of Sir2 NAD-dependent deacetylase activity (137). The 1:1 requirement of NAD^+ for Sir2 deacetylase activity predicts that NAD^+ levels may be a rate-limiting component for its activity. Indeed, nicotinamide clearance by increased expression of PNC1 (pyrazinamidase/ nicotinamidase 1), which encodes an enzyme that deaminates nicotinamide, directly regulates Sir2- activity (148, 149).

1.4.3 Sir functions:

Four further genes were discovered in *Saccharomyces cerevisiae* with high homology to SIR2 (SIR1, SIR3 and SIR4) (129). None of the four genes were essential for life, but they all were involved in silencing at the mating-type loci and telomeres, as well as cell-cycle progression and genomic integrity.

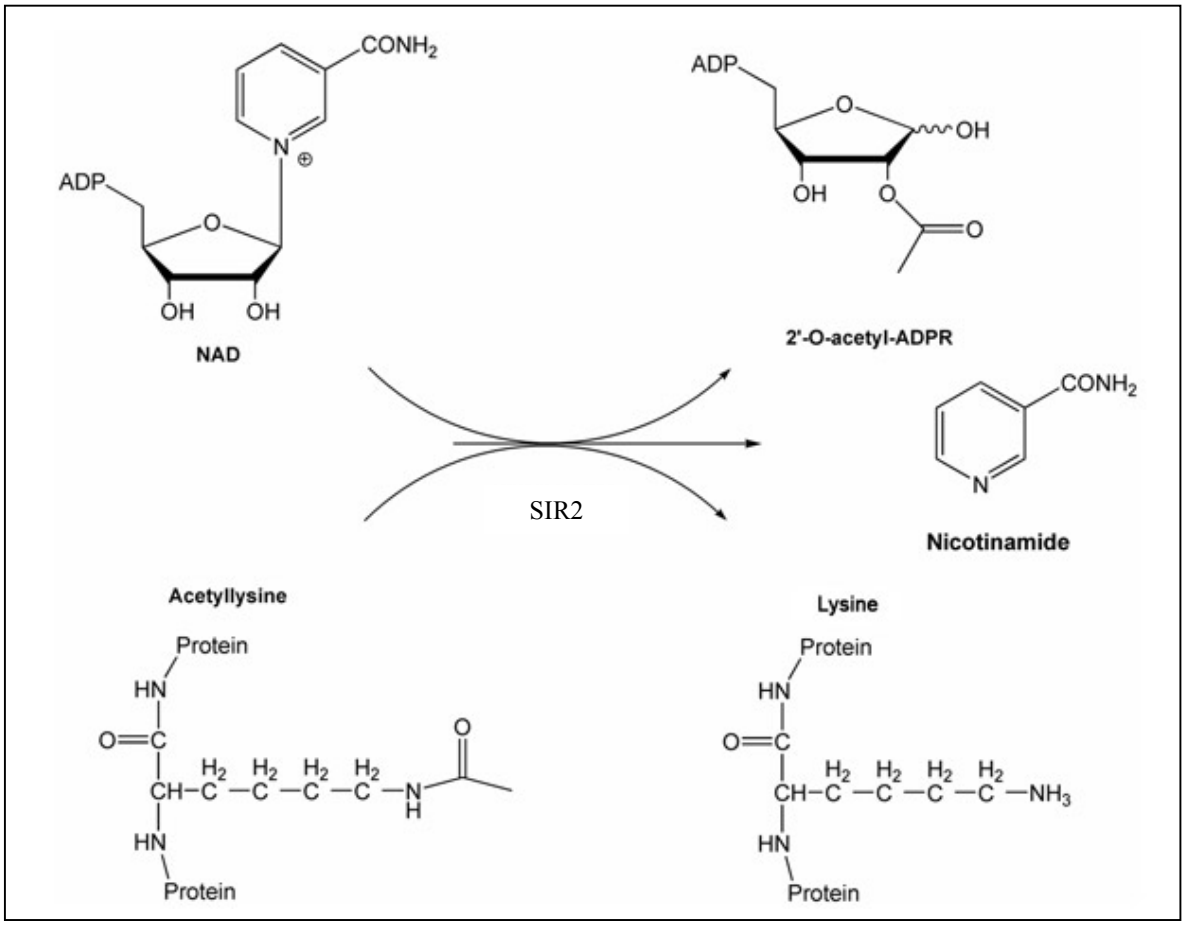

Figure 0.7: Mechanism of SIR2-dependent deacetylation enzymatic activity.

An acetylated protein substrate reacts with one molecule of NAD^+ in the presence of SIR2 (enzyme) to generate one molecule of deacetylated protein, nicotinamide, and 2-O-acetyle-ADP-ribose (modified form (150). This reaction is NAD^+ dependent.

1.4.3.1 Silencing at mating type loci:

Sir2, along with Sir1, Sir3 and Sir4 were first identified as essential components for the transcriptional repression of the silent mating type loci HML (homothallic left) and HMR (homothallic right), in the budding yeast *S. cerevisiae* (8, 151). The mating type in *S. cerevisiae* is determined by the expression of genes located in the active mating locus called MAT. These three separate loci (MAT, HMR, and HML), located on chromosome III, contain either a, or α, mating type genes. Moreover, mating type inter-conversion occurs by transposition of either a, or α, mating type cassettes from the inactive loci HMR, or HML, to the MAT locus. Co-expression of both a and α mating type genes results from loss of silencing at these loci; consequently sterility in haploid strains will ensue (152). In addition to SIR proteins, different elements have been shown to be involved in the silencing process, including silencers, Rap1 (repressor activator protein 1), ORC (origin recognition complex), and Abf1 (Autonomously replicating sequence (ARS) binding factor

1). Two *cis-acting* DNA sequences <250-bp, known as E and I silencers, flank HMR and HML and serve as binding sites for Rap1, Abf1 and ORC complex. The binding of these proteins will recruit the SIR2 proteins, along with other Sir proteins, to the HMR and HMR loci to form a complex, which in turn are responsible for the formation of the heterochromatin structure in the region adjacent to the silencers. Given the deacetylation function of SIR2 protein (136, 153) it will create a specific acetylation pattern in the adjacent nucelosomes, consequently increasing their affinity for the Sir proteins. Afterwards, another Sir complex will bind to the newly modified nucleosome and modify the adjacent one propagating the silenced region (Figure 1.8) (154). Silencers flank the HM loci, demarcate the boundaries of SIR protein deposition, thus preventing repression from being inappropriately disseminated (155, 156). Availability of SIR proteins also contribute to the demarcation of silenced domain (8).

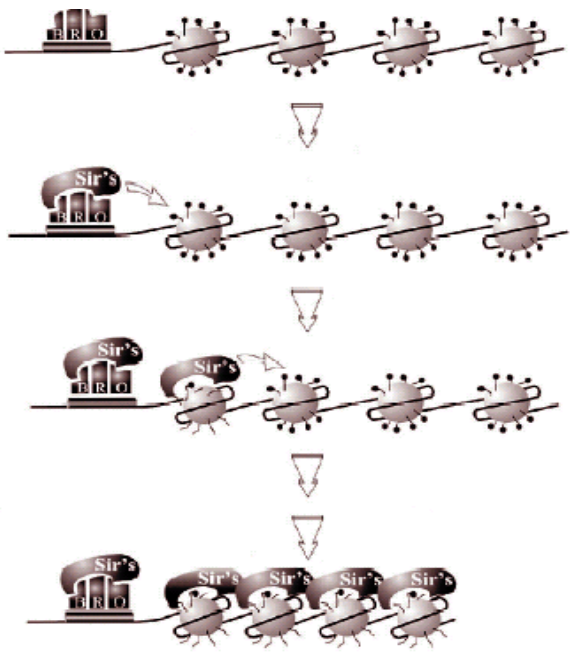

Figure 0.8; A model for silencer-induced heterochromatin formation, and a proposed role for acetylation in silencing.

Rap1(R), Abf1(B), and ORC(O) proteins bind the silencer and recruit additional proteins, SIR2, SIR3,and SIR4 (SIR's) that are themselves responsible for the formation of the heterochromatin structure in the region adjacent to the silencer. These SIR proteins induced a specific pattern of acetylation in the adjacent nucleosome, which increases the affinity of the nucleosome for the SIR proteins. The SIR proteins then bind to the newly modified nucleosome and modify the adjacent one. In this manner SIR proteins can propagate the silencing domain. Modified from (Braunstein et al. 1996).

Sir2 along with Sir3, Sir4, telomere repeated binding protein and Rap1 are associated with the telomere and involved in its silencing in yeast. (157-159). Rap1 binds the terminal telomeric sequence and recruits the Sir proteins, in complex formation for assembling histones to form silent chromatin (160). The suppression extends to involve telomere

proximal genes, in a process known as Telomere Positional Effect TPE (161, 162). Normally, this repression does not extend beyond 3Kb from the telomeric repeat, although in strains over-expressing Sir 3, TPE can extend up to 20 Kb from the chromosomal end (163).

SIR proteins also play a role in preserving both the integrity and sub-nuclear localization of yeast telomeres. The absence of SIR3 or SIR4 not only decreases TPE, but also leads to both telomere shortening and enhanced chromosome loss (162). Silencing of the sub-telomeric region requires Ku protein, a heterodimereic protein that associates with DNA ends. Ku proteins play a role in the integrity of telomere structure, the nuclear localization of chromosome ends, and TPE (164, 165). It interacts with SIR4 and recruits SIR proteins for silencing, in cooperation with Rap1 (166, 167).

1.4.3.2 Sir 2 and DNA repair.

The Sir proteins may also function, along with Ku protein, in DNA double strand break (DSB) repair, in a process known as Non Homologous End Joining (NHEJ) (164, 166, 167). Consistently, it has been shown that deletion of Sir 2, Sir 3, and Sir 4 results in less efficient ligation of DNA, significant decrease in the NHEJ process and increased radiation sensitivity (166-169). Sir proteins relocalize from telomeres to sites of DSB, probably following the initial Ku relocalization from telomeres to the site of damage (169, 170) (Figure 1.9).

1.4.3.3 Suppression of Ribosomal DNA (rDNA) recombination.

Besides its role in NHEJ, HM and telomeric silencing, Sir 2 is also involved in a different mechanism of silencing at rDNA (171, 172). Initially, Sir2, in the absence of Sir3 and Sir 4, was shown to be involved in the suppression of rDNA mitotic recombination, as the frequency of recombination at the rDNA showed a ten fold increase in SIR2 mutants (9). The rDNA locus in yeast is located 450 kb from the left end and 610 kb from the right end of chromosome XII and consists of 100 to 200 tandem repeats copies of a 9.1 kb rDNA unit (173). Each unit contains genes encoding the 35S rRNA and the 5S rRNA, separated by non-transcribed spacer (174-176). The nucleolus is positioned at the periphery of the nucleus, where rDNA transcription, rDNA transcript processing, and pre-ribosomal particles formation take place (177). Immunoflourescence studies have shwon that the majority of Sir2 is located in the nucleolus (178), suggesting an important role. Moreover, Sir 2 has the ability to induce alteration in the rDNA chromatin structure, which is

responsible for transcriptional silencing and suppression of recombination at this locus (171). Furthermore, Net1 protein has been found to be required for Sir2 association with the rDNA locus and for rDNA silencing (179). This protein is a constituent of a complex called regulator of nucleolar silencing and telophase exit, RENT, which is involved in control of the cell cycle (as discussed later in the text).

1.4.3.4 Silent information regulators and life span (Ageing).

Mutation in either of the SIR3 or SIR4 genes result in a 20% decrease in yeast life span, whereas mutation in the SIR2 genes showed a more striking reduction of 50%. This implies that various sirtuins can affect yeast life span differently. Three mechanisms have been suggested to account for this. Firstly, mutation in the SIR3 or SIR4 genes results in derepression of HM and consequently a simultaneous expression of a and α mating type information. This co-expression has been found to be responsible for a small increase in recombination at rDNA, thus increasing the formation of exrtrachromosomal ribosomal DNA circles (ERC), which will shorten the life span (152). Secondly, the loss of homologous recombination inhibition at rDNA, as a direct result of absence of Sir 2 and increased ERC formation are the cause of a significant decrease (50%) in the SIR2 mutant lifespan. This mechanism does not involve mating type silencing, as deletion of HMLα failed in restoring the normal lifespan of the SIR2 mutant, whereas it extends the lifespan in SIR3 or SIR4 mutants. Sir2 acts directly to suppress ERC formation by inhibiting homologous recombination at the blocked replication fork (via Fob1 protein) in the rDNA. Thirdly, the observation that the SIR2 FOB1 HML mutant strain has a shorter life than that of a FOB1 mutant indicates another as yet unknown mechanism plays a role (152).

The level of SIR2 has also been shown to be a limiting factor in yeast ageing, as a second copy of *SIR2* can enhance lifespan over those with only a haploid complement (152). Increased dose has also been shown to increase life span in the nematode *Caenorhabditis elegans* (180).

1.4.3.5 Regulation of cell cycle and meiosis.

Sir2 is involved in cell cycle control through its contribution in forming a complex known as (RENT) regulator of nucleolar silencing and telophase exit. RENT consists of Sir2, Net1, Cdc14, and Nan 1 proteins. Net1 is required for Sir2 association with rDNA and its recombination suppressive function (179). It also inhibits and anchors the mitotic regulator Cdc14 to the nucleolus. Cdc14, in late anaphase, leaves the nucleolus for the cytoplasm

where it reaches its target and gains activity, thus resulting in the cell exiting mitosis (181, 182). The fourth protein Nan1, which is essential in the RENT complex, as yet, has no known function.

Moreover Sir2, in the nucleolus, also functions in controlling a meiotic checkpoint. Checkpoints are protective mechanisms that block cell cycle development when previous, or preparatory, events in the cell cycle have been damaged or, for some reason, not completed properly. The pachytene checkpoint stops meiotic progression in the pachytene stage of the cell cycle, if the cell is defective in chromosome synapsis and recombination. Failure at this checkpoint allows defective yeast cells to proceed through the meiotic cell cycle and to sporulate, producing low viability spores due to chromosome mis-segregation. Pachytene 2 has been identified as a mediator of the pachytene checkpoint. It is tethered to the nucleolus in meiotic cells by SIR2 (183). Furthermore, it has been suggested that the pachytene2-mediated checkpoint involves silent chromatin (8).

1.4.3.6 Sir, caloric restriction, and life span.

Calorie restriction (CR) is a dietary regimen in which an organism is provided with at least 20 % fewer calories than it would naturally consume ad libitum, while maintaining adequate nutrition (184). CR-mediated lifespan extension has been demonstrated in different organisms including yeast(185), fruit flies (D. melanogaster) (186), nematodes (C. elegans) (187), spiders (Frontinella pyramitela) (188) and mice (189). This diversity argues that the mechanisms underlying CR are ancient and well conserved.

This lifespan extension can be explained by the attenuation of oxidative damage and changes in fuel utilisation. The reliance of Sir2 on its NAD co-substrate is central to this lifespan extension. Multiple studies suggested that CR leads to increased mitochondrial NAD levels, reduced NADH levels (190), or reduced nicotinamide levels (191, 192) which in turn activate Sir2.

As a result, CR may result in an increase in the Sir2 activity at telomeres and within the nucleolar rDNA (193), resulting in, a decrease in the oxidative stresses that may also result in a fall in DNA damage and increased concentration of Sir2 at telomeres and the nucleolus. These findings have led to the hypothesis that sirtuins underlie the beneficial effects of caloric restriction in diverse species, including mammals. Further support for this hypothesis has come from studying the mammalian sirtuins (194).

Interestingly, the plant polyphenol, resveratrol, found in large quantities in red wine, has been found to be a potent Sir2 activator and can, like CR, increase DNA stability and extend lifespan in yeast (195). This function of red wine may further explain the 'Mediterranean paradox' of why ageing-related diseases, such as atherosclerotic arterial disease are less common in this red wine drinking population, irrespective of their caloric intake (196, 197).

The diverse functions of Sir2 in yeast are closely linked and reflect the central role that Sir2 plays in growth and proliferation, DNA damage repair and ageing in response to exogenous stresses and nutritional availability. One of the most exciting discoveries to be made in this regard, is that caloric restriction (CR) results in a Sir2-dependent prolongation of lifespan.

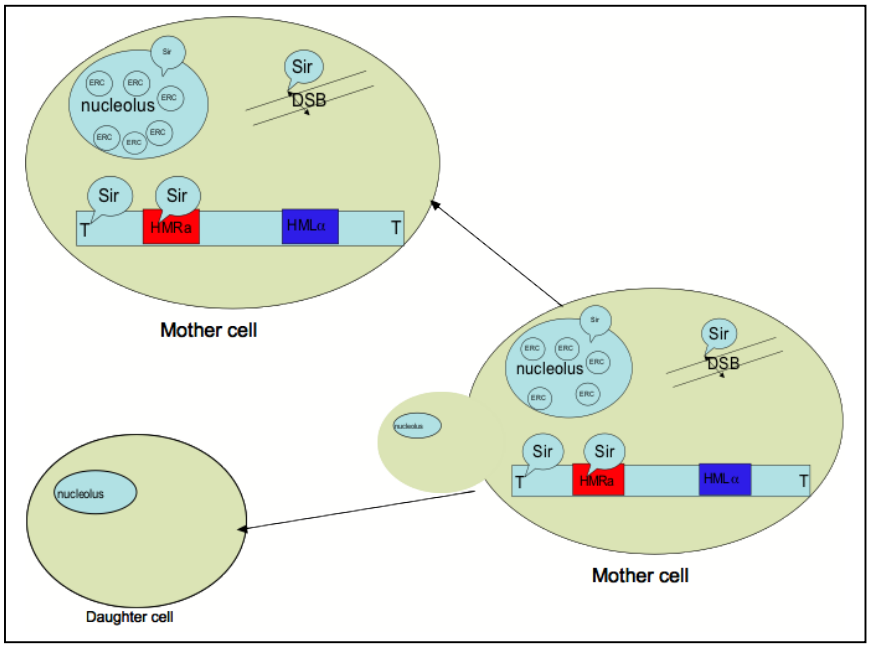

Figure 0.9: The main SIR functions in *Saccharomyces cerevisiae:*

Silencing at the telomere and HML, DNA repair, nucleolar ERC formation and preferential segregation to mother cells. The locations of SIR in *Saccharomyces cerevisiae* are shown together with the preferential segregation of extrachromosomal ribosomal DNA circles (ERC) to the mother cells with cell division.

Sir2 has been shown to relocate to the nucleolus with increasing age, and Sir2 concentration is directly related to life span. Homologous recombination-mediated repair of DSB, occurring within the rDNA, results in the formation of extrachromosomal ribosomal DNA circles (ERC) (198). These structures are segregated in a biased manner towards the yeast mother cell (Figure 1.9). Once accumulation of these ERCs reaches a critical level, the mother cell can no longer divide (199).

1.4.4 Mammalian Sirtuins.

In man, seven Sir2 orthologues termed sirtuins have been characterised, (SIRT1-7) (Table 1.7). These are grouped into four main phylogenetic groups, centred on the characterisation of 60 amino acid 'conserved core domains' from different prokaryotic and eukaryotic sirtuins (127, 130). A fifth class has been added based on *Firmicute* (gram positive) bacteria and *Thermotoga maritima* sirtuin proteins, with sequence motifs that seem to be intermediate between classes II and III and the classes I and IV. This form of sirtuin has been classified as group U.

In man, the seven sirtuins represent the four known classes. SIRT1, SIRT2, and SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and SIRT7 to class IV (127). Mammalian sirtuins also differ in their sub-cellular localization. SIRT1, SIRT6 and SIRT7 are predominately in the nucleus (although SIRT1 does have some important cytoplasmic functions as well). In the nucleus a large fraction of SIRT1 is associated with euchromatin, whereas SIRT6 associates with heterochromatin and SIRT7 is found in the nucleolus (200). The sirtuin that resides most prominently in the cytoplasm is SIRT2 (7, 201).

SIRT3, SIRT4 and SIRT5 have been described as mitochondrial sirtuins (200, 202, 203).

These mammalian sirtuins differ also in their enzymatic activity:

- SIRT1 and SIRT5 only exhibit robust and weak deacetylase activity respectively (146, 201).
- SIRT4 is only a mono- ADP-ribosyl tranferase (204).
- SIRT2, SIRT3 and SIRT6 appear to have both deacetylase and mono-ADP-ribosyl tranferase activities (7, 201, 205-207)
- SIRT7 has NAD deacetylase activity.

Each of these sirtuins will be described in more detail below.

Table 0.7: Classification of human sirtuins.

The table shows the class, location, molecular weight, enzymatic function and biochemical features of the human sirtuins and their genes.

Sirtuin	Class	chromosome	Cellular location	Transcript(s) length (base pairs)	Amino acid residues	Molecular weight (kilodaltons)	Enzymatic function
SIRT1	I	10q21.3	Nucleus, Cytoplasm	4086	747	82	HDAC,
SIRT2	I	19q13.2	Cytoplasm	1963, 1931	399	43	HDAC, ADP-ribosyl tranferase
SIRT3	I	11p15.5	Mitochondrion	2900, 2574	399	44	HDAC, ADP-ribosyl tranferase
SIRT4	II	12q24.23	Mitochondrion	1163	314	35	ADP-ribosyl tranferase
SIRT5	III	6p23	Mitochondrion	2350, 1670	310	34	HDAC
SIRT6	IV	19p13.3	Nucleus	1638	355	39	HDAC, ADP-ribosyl tranferase
SIRT7	IV	17q25	Nucleus	1718	400	45	HDAC

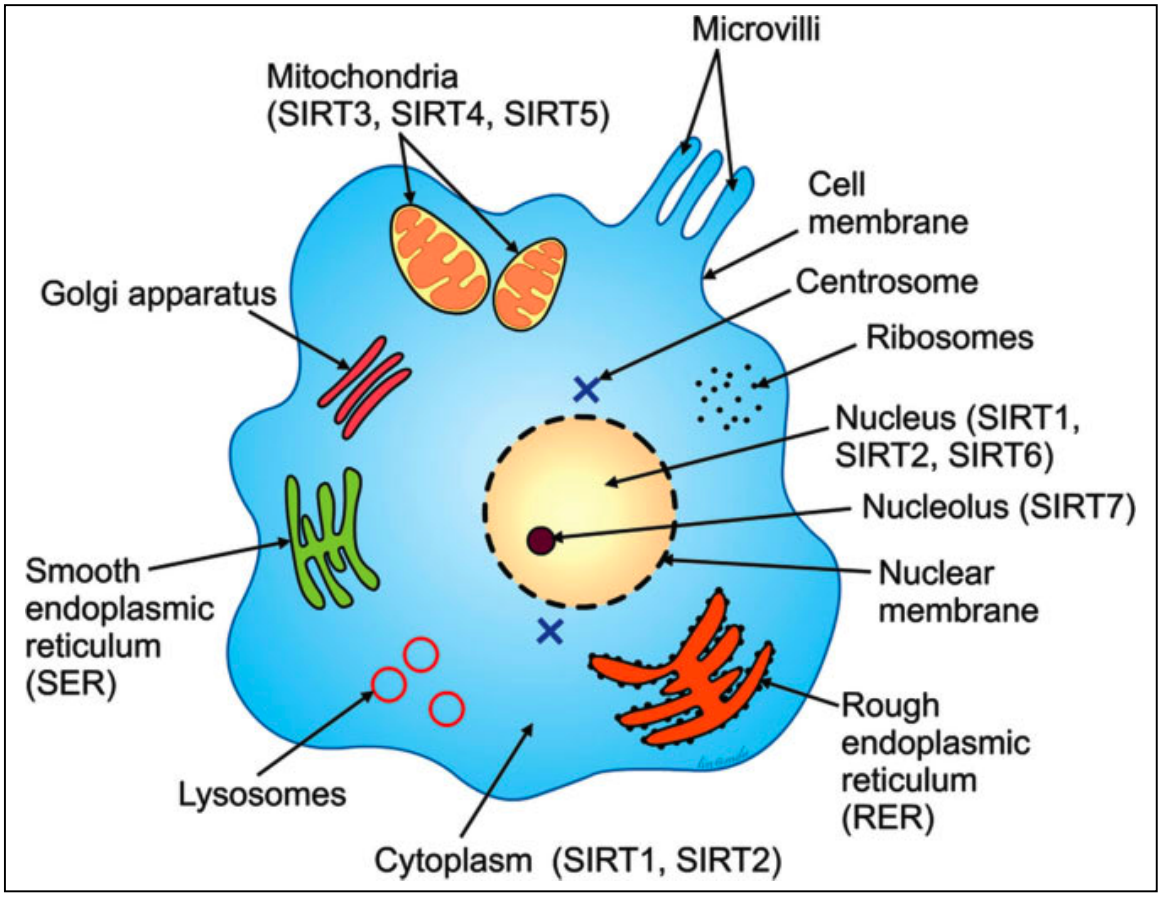

Figure 0.10: Intracellular location of sirtuins.

SIRT1 may be present in the nucleus and/or cytoplasm, depending on the type of biological tissue. SIRT2 is found in the cytoplasm, and is able to shuttle to the nucleus. SIRT3, SIRT4, and SIRT5 are present in the mitochondria whereas SIRT6 and SIRT7 are present in the nucleus and nucleolus, respectively (208).

1.4.4.1 Mamalian Sirtuin1.

The human sirtuin1 (SIRT1) gene has been mapped to chromosome 10. It is considered the human homologue of the yeast *SIR2* gene (7). Initially SIRT1 has a postulated nuclear localization similar to yeast Sir2, based upon a putative nuclear localization signal coded by amino acids 41-46 (7). Mammalian SIRT1 (Figure 1.5) has a long N-terminal sequence that shows little conservation with yeast Sir2, but contains functional nuclear localization sequences that are conserved among SIRT1 from different mammals. Together with nuclear exporting sequences, located in the catalytic domain, the nuclear localization sequences control the nucleus-cytoplasm shuttling of SIRT1 (209)

Similar to yeast Sir2, mammalian Sirt1 exhibits predominantly nuclear localisation and facilitates the formation of heterochromatin that is associated with histone hypoacetylation and gene repression. SIRT1 targets for deacetylation include lysine residues at positions 9 and 26 of histone H1, 14 of H3, and 16 of H4 (136, 210). Multiple non-histone targets have also been described including p53 (144, 146), p300/CBP-associated factor (PCAF) (211), p300 (212), MyoD (213, 214), the Foxo transcription factors (215-217), NF-κB (218) and E2F1 (219). In addition, SIRT1 interacts with the HES1/HES2 factors, chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2, CTIP-2 and (Peroxisome proliferator-activated receptor- γ) PPAR-γ (220, 221). Interaction of SIRT1 with these substrates, allows SIRT1 to be recruited to chromatin regions and specific promoters, to influence the transcription of other genes, leading to the involvement of SIRT1 in a wide variety of important cell processes, including apoptosis (144, 146, 211) development and embryogenesis (213, 221-224), glucose and fat metabolism (225-227) axonal degeneration (228) and muscle differentiation (213, 229). The involvements of SIRT1 in these cellular processes will be explored in the following sections.

1.4.4.1.1 Posttranscriptional regulation of Sirtuin1.

SIRT1 expression is also regulated at the posttranscriptional level. The RNA binding protein HuR regulates the stability of many target mRNAs. It was recently reported that HuR associated with the 3' untranslated region of SIRT1 mRNA stabilized the mRNA and increased SIRT1 expression (230). This study found that oxidative stress triggered the dissociation of the HuR from SIRT1 mRNA, in turn promoting SIRT1 mRNA decay, reducing SIRT1 abundance and lowering cell survival. This occurs by the activation of the cell cycle checkpoint kinase, Chk2, which interacts with HuR and possibly phosphorylates

HuR, an event that appears to be important for HuR dissociation after hydrogen peroxide treatment.

1.4.4.1.2 Sirtuin1, cell survival and apoptosis.

SIRT1 is thought to play an important regulatory role in mediating cell survival by preventing p53-mediated growth arrest, senescence and apoptosis. SIRT1 mediates apoptosis in response to stimuli including oxidative damage, γ-radiation and exposure to tumour necrotizing factor alpha (TNFα) (144, 146, 231). SIRT1 also negatively controls p73- mediated apoptosis (232).

1.4.4.1.2.1 Sirtuin1 and p53.

P53, short lived tumour suppressor protein, is the best known gatekeeper tumour suppressor that induces anti-proliferative effects, including growth arrest, apoptosis, and cell senescence, in response to different kinds of stress (233-235). Three separate studies have shown that p53 activity is negatively regulated by SIRT1-mediated deacetylation (144, 146, 231). Strict control of p53 activity is essential to regulate normal development in addition to tumour suppression. This control is generally thought to involve a post-translational modification of p53 (236). p53 is normally phosphorylated in response to DNA damage, by means of kinases, such as Ataxia telangiectasia mutated (ATM) (237). This phosphorylation does not lead directly to activation of p53, but increases p53 affinity for p300 acetylase, eventually leading to acetylation and activation of p53 (238). p53 acts as a transcription factor, and upregulates the expression of cycline dependent kinase inhibitor p21 that promotes cell cycle exit (239). Alternatively, p53 activation may, depending on the degree of DNA damage, upregulate the expression of genes that favour apoptosis (240).

Conversely, if the DNA damage has been successfully repaired, a p53 inhibitory system would be needed to regain active cellular growth. SIRT1 can selectively bind and deacetylate p53, thus inhibiting its activity. This will impede the p53-dependent apoptosis response, or relieve the blockage of cell cycle imposed by p53-dependent p21 up regulation.

Under normal circumstances, murine double minute 2 (Mdm2) is the major negative regulation of p53 (241) and is able to block its acetylation by p300 (242). Deacetylated p53 is unstable and is rapidly degraded through Mdm2-mediated ubiquitination. Thus Mdm2 maintains p53 at low levels to enable the cell to grow normally. If DNA is damaged during

stress, p53 will undergo phosphorylation, a prerequisite for acetylation, which prevent the binding of Mdm2 (236).

However, in a stressed cell, acetylated p53 resists Mdm2-mediated ubiquitination, a process that results in protein degradation. This might be the result of acetylation of the same site required for Mdm2-mediated ubiquitination (243, 244). Consequently, highly acetylated p53 cannot be effectively degraded by Mdm2 until it is deacetylated (245). Moreover, ATM also phosphorylates Mdm2 decreasing its inhibitory potential on p53 (246). Therefore, in DNA damaged cells, the main p53 regulatory pathway, Mdm2-mediated, is blocked at several levels. Thus SIRT1 may play an important role in negatively controlling p53 activity, as the other regulator is inactive. ATM, like its yeast mec1 kinase counterpart responsible for the redistribution of the yeast sir complex to areas of DSB to facilitate their repair, may also play a similar role in mobilising SIRT and other repair machinery to damaged DNA in mammals.

SIRT1 has also been shown to have binding sites for p53 and p53 can itself stimulate SIRT1 transcription, in a Foxo-dependent manner (247). Another regulatory mechanism for SIRT1/p53 has been described in co-operation with the hypermethylated in cancer 1 transcriptional repressor (HIC1)(248). SIRT1 mediates a bypass of apoptosis, possibly by promoting cell survival, via formation of a complex with HIC1, which binds directly to the SIRT1 promoter and represses its expression, thus promoting p53-dependent apoptotic responses. Since p53 is able to transactivate HIC1 transcription, it has been proposed that SIRT1—HIC1—p53 act in a complex feedback loop. Under normal physiological conditions, HIC1 represses SIRT1, promoting p53 activity and apoptosis under stress. However, in cells set to recover from DNA damage, p53 down-regulates HIC1, which result in the induction of SIRT1 transcription and promotes cell survival (248). The authors of the study speculate that HIC1 hypermethylation experienced during ageing may lead to increased SIRT1 expression, which may deacetylate p53 and increase cellular risk for neoplastic transformation and tumourigenesis.

There is evidence for several other regulatory negative feed back loops acting on SIRT1:

- DeltaNp63alpha, a p53 related protein, down-regulates SIRT1 and accelerates the ageing phenotype when over-expressed in mice (249).

- Deleted in Breast cancer 1 protein (DBC1) antagonises SIRT1 deacetyaltion of p53 resulting in increased levels of acetylated p53 and up regulation of p53-mediated

function in human cells (250). Elimination of endogenous DBC1 by RNA interference (RNAi) stimulates SIRT1-mediated deacetylation of p53 and inhibits p53-dependent apoptosis. (250).

In response to DNA damage, SIRT1 may therefore play an important regulatory role in mediating cell survival by deacetylating and thereby inactivating p53, preventing growth arrest, senescence or apoptosis. Levels of SIRT1 might be informative of cellular levels of stress and indicative of p53 status. Furthermore, SIRT1 could be an important determinant of sensitivity and play a role in dictating the response to antitumour agents, especially those that damage DNA.

1.4.4.1.2.2 Sirtuin1 and FOXO transcription factors.

Another alternative pathway by which SIRT1 increases cell survival is through the regulation of Foxo transcription factors. There are four Foxo proteins and so far SIRT1 has been shown to deacetylate three of them: Foxo1, Foxo3a and Foxo4. The Foxo factors induce the transcription of a wide variety of genes involved in the oxidative stress response, DNA repair, cell-cycle control and apoptosis (251). In response to oxidative stress, SIRT1-mediated deacetylation of the Foxo forkhead family of transcription factors also occurs (215, 217, 252-255). The effects of SIRT1 on Foxo-controlled genes vary between tissues and range from their activation to repression. Overall, it appears that SIRT1 activity results in a decrease in Foxo3-mediated apoptosis, an increase in Foxo3-mediated DNA repair and cell-cycle arrest and an increase in both Foxo1 and Foxo4-mediated stress resistance (215, 217, 254, 256, 257). In addition, it has been shown that SIRT1 acting via Foxo4 suppresses the pro-apoptotic proteases caspase-3 and caspase-7 in transformed, but not in untransformed epithelial cells (258). In what appears to be a feedback loop, caspase-9 and Bcl-xL regulate SIRT1 cleavage during apoptosis, shifting its localization from the nucleus to the cytoplasm (249).

The overall effect of SIRT1 action is to shift Foxo-induced responses away from apoptosis towards cell-cycle arrest, perhaps allowing cells time to repair the DNA damage and detoxify free radicals. Through its interaction with the Foxo factors, SIRT1 mediates stress resistance in a p53-independent manner. Evidence supporting this comes from the observation that apoptosis in cells lacking SIRT1 is only partially rescued by transfection with a dominant negative *p53* mutant (146) and that in cells lacking functional p53, the SIRT1 activator, resveratrol, can still increase resistance to γ–radiation (195).

1.4.4.1.2.3 Sirtuin1 and NF-κB.

In contrast to the cell survival promoting effects described previously, SIRT1 can exert a pro-apoptotic effect through its interaction with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a protein complex that acts as a transcription factor. NF-κB is found in almost all animal cell types and is involved in regulating genes that control cell survival, and in cellular responses to stimuli such as stress cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens. NF-κB appears to be an important regulator of ageing-related cellular processes. It exists as a heterodimer, composed of p50 and RelA/p65 polypeptides. Within the cytoplasm it is normally bound to members of the I-κB family of inhibitory proteins. Once activated NF-κB is released into the nucleus where it enhances transcription by tethering histone acetyltransferases (HAT) that result in an open chromatin structure. Histone deacetylases (HDAC) including SIRT1 inhibit NF-κB transcription (218, 259).

NF-κB activity is controlled by multiple layers of regulation (260). All five members of the NF-κB family contain a Rel-homology DNA binding domain. These NF-κB proteins typically bind to their target DNA sequences as dimers. In unstimulated cells, NF-κB is sequestered in the cytoplasm by inhibitory proteins (I-κB). Upon stimulation by diverse cell stresses, I-κBs are degraded, allowing NF-κB to translocate into the nucleus and activate target genes. NF-κB induces the transcription of IκBα and other negative regulators of the pathway, which contribute to signal inactivation. Thus, in individual cells, NF-κB signalling is characterized by dynamic patterns of periodic NF-κB nuclear localization and target gene activation interspersed with nuclear exit and gene deactivation. (260).

Previously, we described how SIRT1 could provide survival advantage by binding, deacetylating and thereby decreasing p53, Foxo and Ku70-mediated apoptosis, however it is also known to deacetylate and inactivate NF-κB, thereby sensitising cells to (TNFα) induced apoptosis. SIRT1 does this by associating with and deacetylating RelA/p65 R, a subunit of NF-κB. Deacetylation inhibits the transactivation potential of RelA/p65, thus sensitizing human cells to apoptosis in response to TNFα (218, 259). Treatment with, resveratrol, a sirtuin activator and known inhibitor of NF-κB transcription, results in a reduction in the transcription of genes, including the anti-apoptotic cIAP-2 gene, following TNFα stimulation. The cIAP-2 gene product inhibits TNFα-induced caspase activation and therefore resveratrol results in sensitisation to TNFα-induced

apoptosis. This effect is SIRT 1-dependent, as loss of SIRT1 expression completely inhibits this TNFα-induced apoptosis (218).

Another study showed that the breast cancer associated protein, BCA3, when neddylated (modified by Nedd8), interacts with SIRT1 and suppresses NF-kB-dependent transcription, also sensitizing human breast and prostate cancer cells (such as MCF-7 and DU145 cells respectively) to TNFα-induced apoptosis (261).

From the previous two sections, it could be argued that SIRT1 plays two different and contradictory roles in controlling cell survival.

Consistent with the pro-survival effect of SIRT1 Kuzmicgev et al. (2005) found that SIRT1, together with other components of Polycomb Repressive Complex (PRC), a complex that maintains homeotic gene repression during development, are over-expressed in colon, breast and prostate cancers as compared to normal tissue controls. (262)

1.4.4.1.2.3.1 SIRTuin1 and inflammation.

SIRT1 might be involved in modulating the inflammatory response, The pro-inflammatory cytokine release is regulated through its interaction with NF-kappaB. It has been shown that cigarette smoke extracts decrease SIRT1 activity, reducing its interaction with RelA/p65 and increaseing NFκB-dependent release of pro-inflammatory mediators (263).

1.4.4.1.2.4 Sirtuin1, Ku70 and Bax-induced apoptosis.

SIRT1 plays a role in cell survival by deacetylating Ku70, a DNA repair factor which is induced by CR (264). Ku70 is a protein, which in humans is encoded by the XRCC6 gene. Together, Ku70 and Ku80 make up the Ku heterodimer, which binds to DNA double-strand break ends and is required for the non-homologous end joining (NHEJ) pathway of DNA repair. The p53 apoptotic response is multifaceted, as it activates numerous pro-apoptotic genes, including Bax, a proapoptotic member of the Bcl-2 family, whose localization to mitochondria triggers cytochrome c release and apoptosis and p53 up-regulated modulator of apoptosis (PUMA), another pro-apoptotic member of the Bcl-2 protein family. Bax is normally rendered inactive by its tight association with the Ku70 protein, leading to sequestering of Bax in the cytoplasm. In response to acute cell damage or stress, Ku70 is acetylated at two lysines (K539 and K542) by the CREB-binding protein (CBP) and the acetyltransferase, p300/CBP-associated factor (PCAF), disrupting the Ku70-Bax association. The Bax protein then initiates apoptosis by re-localizing from the

cytoplasm to the outer mitochondrial membrane. The result is the release of cytochrome and downstream events including caspase activation (211, 265). SIRT1 deacetylates Ku70 at the K539 and K542 sites. Such an action strengthens the ability of Ku70 to sequester Bax away from mitochondria and inhibits apoptosis mediated by Bax.

CR induced SIRT1 expression protected embryonic kidney stem cells (293T cells) from Bax-induced apoptosis (142). Although the main pro-survival function of SIRT1 is attributed to its nuclear action, a recent study (266) showed that SIRT1 is cleaved by caspase during the apoptosis of N2a neuroblastoma cells, which is associated with increased cytoplasmic localization of SIRT1. The data suggest the possible involvement of cytoplasmic action of SIRT1 in cell death. The studies however did not make it clear whether the cleavage and increased cytoplasmic localization is a consequence of cell apoptosis, or a cause. It is also unclear whether the cleavage product still functions as a NAD+-dependent deacetylase.

1.4.4.1.2.5 Sirtuin1, E2F1 and Retinoblastoma protein.

The E2F family of transcription factors have roles in regulating cell proliferation and apoptosis in higher eukaryotes and their activity is controlled by the retinoblastoma protein (pRb) tumour suppressor during the cell cycle (267). The pRb protein has a crucial role in regulating the G1- to S-phase transition, and its phosphorylation by cyclin-dependent kinases is an established and important mechanism in controlling pRb activity. In addition, the targeted acetylation of lysine (K) residues 873/874 in the carboxy-terminal region of pRb located within a cyclin-dependent kinase-docking site impedes pRb phosphorylation and thereby retains pRb in an active state of growth suppression (268). Besides cell cycle regulation, some E2F members, particularly E2F1, also have important roles in regulating apoptosis. For instance, E2F1 stimulates the transcription of several genes in the apoptotic pathways (267) and its over-expression induces premature S-phase entry and apoptosis (269, 270). Similar to ATM and p53, DNA damage results in stabilization of E2F1 thus inducing apoptosis. E2F1 might be involved in the apoptotic respond induced by etoposide (a chemotherapeutic drug clinically used for treating patients with testicular cancer, or small cell lung cancer) in a non-small cell lung carcinoma cell line (H1299) (219, 271). SIRT1 deacetylated E2F1, suppressed its transcriptional activity and pro-apoptotic function (219). Interestingly, there is a negative feedback loop between E2F1 and SIRT1, as E2F1 induces SIRT1 expression, which binds to and inhibits E2F1 activity (219). Inhibition of SIRT1 results in an increase in E2F1-mediated apoptosis. SIRT1 may prevent E2F1-

mediated apoptosis, thereby allowing DNA damage repair. The interaction between SIRT1 and E2F1 suggest an involvement of SIRT1 in resistance to chemotherapy.

Furthermore, pRb can also be negatively regulated by SIRT1. A recent study (272) showed that SIRT1 can directly interact with and deacetylate pRb, thus inactivating the growth suppression effect of pRB in both *in vitro* and *in vivo*.. Furthermore, the deacetylation action of SIRT1 on pRb was dependent on NAD and inhibited by the SIRT1inhibitor nicotinamide (272).

1.4.4.1.3 Sirtuin1, growth and differentiation.

Another aspect of SIRT1 controlling cellular fate could be the effect of SIRT1 on cellular terminal differentiation. SIRT1 has a physiological role in regulating gene expression and differentiation in muscles by sensing changes in the [NAD]/[NADH] ratio. SIRT1 suppresses myoblast differentiation by deacetylating and inhibiting the transcription factor MyoD (209). SIRT1 is able to inhibit skeletal muscle gene expression and differentiation. This is achieved by SIRT1 enhancing the transcriptional repression of Hes1 and Hey2, members of Hairy-related protein sub-families (221). These are transcriptional repressors that prevent cellular differentiation (273). Finally, SIRT1 may also prevent muscle differentiation by associating with and deacetylating Pcaf, thereby causing an increase in the interaction between Hey1 and the muscle differentiation transcription factor, MyoD, Furthermore, SIRT1 directly represses the action of MyoD through direct interaction with the two MyoD regulated promoters, MHC and myogenin (213). In skeletal muscles, SIRT1 also regulates metabolic changes through deacetylation of PGC-1a, which is required for activation of mitochondrial fatty acid oxidation genes (213). Increased levels of SIRT1 are also seen in satellite muscle cells from older animals. These muscle cells play a role in the regeneration and repair of damaged muscle (274). In aged rats, there is lower muscle satellite cell proliferation and in a study looking at the protein expression in muscle cells from older rats, p21, p53, SIRT1 and Foxo1 were all found to be higher than in cells from younger animals (229). Rather than controlling muscle cell growth, increased SIRT1 in older animals may reflect the cell's attempt at limiting the p53 and Foxo-mediated apoptosis in muscle cells, as a result of ageing.

SIRT1 inhibits androgen and regulates the function of the androgen receptor by deacetylation and transcription, thus influencing muscle mass (232, 275). It also acts as a major repressor of uncoupling protein-3 (Ucp-3), which plays a role in muscle cell protection against overload of fatty acids, lowering mitochondrial membrane potential and

reducing production of excessive reactive oxygen species (276). SIRT1 has also been shown to inhibit androgen receptor dependent cell proliferation in prostate tumour cells (277)

1.4.4.1.4 Sirtuin1, Glucose and fat metabolism.

Skeletal muscle is also the primary site of insulin-mediated glucose uptake and SIRT1 in muscle tissue may influence glucose uptake in response to food intake, fasting and exercise. In pancreatic β cells of transgenic mice that over-express SIRT1, there is an improvement in glucose tolerance and enhanced insulin secretion in response to glucose stimulation (225). SIRT1 does this by repressing expression of the mitochondrial uncoupling protein Ucp-2 (278). SIRT1 deacetylation of Foxo1 also promotes resistance to oxidative stress in pancreatic β cells (279).

SIRT1 modulates hepatic gluconeogenesis and glycolysis in response to fasting. In this situation, SIRT1 expression increases in a Foxo3a and p53-dependent manner (247). SIRT1 then deacetylates and decreases the expression of the mitochondrial biogenesis co-activator Pgc-1α, a regulator of cellular metabolism in response to fasting (226). SIRT1 thereby induces gluconeogenic genes and glucose release and modulates Pcg-1α mediated repression of glycolytic genes (227).

Genes regulating fat development and mobilisation are also under the control of SIRT1. In response to food deprivation, SIRT1 promotes fat mobilisation in white adipocytes by binding to and repressing genes controlled by the fat regulator, peroxisome proliferator-activated receptor-γ, PPAR-γ. It does this by binding with its cofactors, nuclear receptor co-factor, NcoR, and the silencing mediator of retinoid and thyroid hormone receptors, SMRT. The result is the mobilisation of fatty acids from white adipocytes and decreased lipogenesis and fat storage. Over-expression of SIRT1, as well as treatment with resveratrol results in an increased SIRT1-mediated reduction in fat (220).

SIRT1 also deacetylates the metabolic enzyme Acetyl Co-Synthetase 1, AceCS1, resulting in an increase in Acetyl-CoA synthesis from free acetate (280). Although the significance of this Acetyl-CoA synthesis is unclear, one of its uses may be in fat metabolism.

1.4.4.1.5 Sirtuin1 and embryonic development.

Multi-cellular organisms employ apoptosis widely during embryogenesis and have adapted this mechanism to rid the organism of redundant tissue during organogenesis. The

expression of SIRT1 is particularly high during embryogenesis. In animal studies, SIRT1 levels were highest in early embryogenesis and, in particular, in embryonic heart and neural tissue. Furthermore, transgenic mice deficient in SIRT1, display cardiac and neurological defects (222, 223).

SIRT1 enhances the transcriptional repression of the Hes1 and Hey2 transcription factors. Hes1 and SIRT1 are both expressed in neural precursor cells and the expression of both proteins decreases during neurogenesis (224, 281). Hes1 is thought to prevent neural differentiation, and therefore it may be speculated that down-regulation of both Hes1 and SIRT1 may be necessary for normal neural development. Hey2 is also detectable in the primitive ventricle during embryogenesis and is thought to regulate its morphogenesis (282). Here too, SIRT1 may interact with Hey2 to perform this function. SIRT1 may affect organogenesis by either fulfilling an anti-apoptotic function or by regulating the transcription of other genes that control growth and differentiation.

1.4.4.1.6 Sirtuin1 and Axonal degeneration.

Axonal degeneration is an active process and is involved in physiological and pathological conditions including Alzheimer's disease, Parkinson's disease and nerve injury (283). NAD and SIRT1 have been shown to prevent axonotomy-associated axonal degeneration (228). Treatment with sirtuin inhibitors prior to injury decreased this protection and, pre-treatment with the SIRT1 activator resveratrol, replicated the axonal protection seen with NAD.

1.4.4.1.7 Sirtuin1 and Haematopoeisis.

SIRT1 is also implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2, (CTIP-2) (284). CTIP-2 itself enhances the transcriptional repression of COUP-TF. The CTIP proteins were originally identified in cells of lymphoid origin and are implicated in normal haematopoietic cell development. Moreover, the CTIP proteins are implicated in the aetiology of haematopoietic malignancies.

1.4.4.2 Mammalian Sirtuin2.

The human sirtuin2 (SIRT2) gene is located on the long arm of chromosome 19 (Table 1.7), and its encoded protein has a cytoplasmic localization (Figure 1.10) (140, 285). Human SIRT2 protein shows a NAD-dependent deacetylase activity (7, 200, 208).

Recently, it has been shown to deacetylate the α-tubulin subunit of microtubules (201, 286).

Microtubules are formed by the polymerization of α and β-tubulin units and play an important role in cell shape, motility, intracellular transport and cell division (287). Many post-translational modifications of microtubules are known to occur, including both acetylation and deacetylation (287, 288).

1.4.4.2.1.1 Sirtuin2, cell cycle and mitosis.

SIRT2 plays an important role in mitosis and cell cycle control. This has been suggested by the dramatic increase of SIRT2 within the cytoplasm during mitosis. In fact, hyperphosphorylated, activated, forms of SIRT2 are present only in the M phase of the cell cycle, at the G2/M phase transition. Over-expression of these enzymatically active forms of SIRT2 results in delay in cell cycle progression. It is hypothesised that deacetylation of acetylated microtubules in the spindle apparatus results in arrest during cytokinesis (201). Furthermore, the CDC14B phosphatase, released in late M phase, may act upstream of SIRT2 by indirectly targeting it for turnover by the 26S proteosome (286). This phosphatase would therefore negatively regulate the SIRT2-mediated delay in cell cycle progression, thereby allowing cell cycle progression. Moreover, SIRT2 also influences microtubule dynamics by affecting the redox potential (213). SIRT2 may therefore participate in a late mitotic checkpoint to ensure correct chromosome segregation during cytokinesis. This is a role consistent with the previously defined relationship between SIR2 and regulation of the cell cycle in yeast (179, 182).

SIRT2 has also been shown to interact with the homeobox transcription factor 10 (HOXA10) suggesting a role for it in mammalian development (289). SIRT2 is expressed in both fetal and adult skeletal muscles, regulating the cell cycle during cell differentiation. Many post-translational modifications of microtubules are known to occur including both acetylation and deacetylation (287, 288). Finally, nucleo-cytoplasmic shuttling of SIRT2 also occurs in response to ionising radiation suggesting a possible role for it in the DNA damage response (290).

As well as controlling mitotic exit, by modulating microtubule dynamics, a putative role for SIRT2 in controlling intracellular transport and motility could be suggested, based upon SIRT2-mediated cytoskeleton control.

1.4.4.3 Mammalian Sirtuin3.

The human sirtuin3 (SIRT3) gene is located on the short arm of chromosome 11. Its encoded protein, SIRT3, has been shown to be located within the mitochondrial matrix (Figure 1.10), and shows both ADP-ribosyl transferase and NAD-dependent deacetylase activity (Table 1.7) (202, 203).

SIRT3 is a major mitochondrial deacetylase in mice: SIRT3-deficient animals exhibit increasing levels of mitochondrial protein hyperacetylation (291). This however has no major impact on metabolism, as SIRT3-deficient mice are metabolically unremarkable. In contrast, no mitochondrial hyperacetylation was detectable in mice lacking the two other mitochondrial sirtuins, SIRT4 and SIRT5 (291).

SIRT3 is initially synthesized within the cytoplasm as a inactive precursor, and transported to the mitochondrial matrix where it acquires its enzymatic activity through a proteolytic process (203). Activated SIRT3 is able to deacetylate and thereby activate the mitochondrial matrix protein, acetyl-CoA synthetase 2 (AceCS2). The cell requires acetyl-CoA as an intermediate for the Krebs cycle, as well as other metabolic processes, including lipogenesis. Although acetyl-CoA is ordinarily produced via the enzymatic conversion of pyruvate, the production of acetyl-CoA by AceCS2 is particularly high during ketogenic conditions such as prolonged fasting, or diabetes.

Indeed, the abundance of AceCS2 within heart and skeletal muscle under ketogenic conditions suggests, that SIRT3 may play an important role in AceCS2 activation and acetyl-CoA production (292). Substantiating this, high levels of SIRT3 expression are found in metabolically active tissue such as muscle, liver, kidney and heart (202).

This mitochondrial localization of SIRT3 is intriguing for two reasons. Firstly, the mitochondrion is the bioenergetic and metabolic centre of eukaryotic cells. Secondly, the conserved family of Sir2 proteins have been postulated to be involved in sensing cellular energy and the redox state of the cell through their NAD-dependent activity, which links their functions including DNA repair, cell cycle regulation, and the control of ageing to cellular metabolism (2, 293, 294). However, SIRT3 might differ from other Sir2 proteins in its sensitivity to metabolic activity, as the level of NAD within the mitochondrial matrix is believed to be stable and not affected by changes in ATP level variations. This might ensure constitutive SIRT3 deacetylation activity (203).

However, an abrupt decrease in mitochondrial NAD level can result from opening of the mitochondrial permeability transition pore (MPTP) opening, which is caused by apoptotic stimuli, reactive oxygen species (ROS), or calcium elevation. This will result in inhibition of SIRT3 activity. MPTP opening will not only cause a rapid decrease in the NAD within the mitochondrial matrix, but will also produce nicotinamide, a known inhibitor of SIR2-like protein activity, as a result of hydrolysis of NAD in the intermembrane space by NADase. In contrast, CR and cold exposure has been reported to be result in upregulation of SIRT3 in brown fat (207).

SIRT3 might play a role in the mitochondrial apoptotic pathway. SIRT3 has been shown to possess a pro-apoptotic function in several cell lines (295). Both SIRT3 expression and its mitochondrial localization increase after Kaempferol treatment in human leukemia cells (296). Kaempferol (3, 4', 5, 7-tetrahydroxyflavone) is a flavonoid with anti- and pro-oxidant activity that has anticancer properties through the induction of the apoptotic programme (296). This study showed that oxidative stress induced by kaempferol in leukemia cells lines causes the activation of the mitochondrial phase of the apoptotic programme with increased Bax and SIRT3, decreased Bcl-2, release of cytochrome c, caspase-3 activation, and cell death, suggesting a role of SIRT3 in apoptosis.

A recent study demonstrated that SIRT3 acts as endogenous negative regulator of cardiac hypertrophy, and protects the heart by suppressing cellular levels of ROS. It has been shown that SIRT3 can protect the mouse heart by blocking the cardiac hypertrophic response through activation of the Foxo3a-dependent, antioxidant-encoding genes manganese superoxide dismutase (MnSOD) and catalase (Cat), thereby decreasing cellular levels of ROS (297).

In view of the evidence presented above, SIRT3 may also act as a sensor of the redox state of the cell, permitting the cell to regulate metabolism, DNA repair, cell-cycle control, apoptosis and ageing in response to oxidative stimuli (294, 298). This possible role for SIRT3 is further supported by a recent study identifying SIRT3 as a novel SIRT1 protein interaction partner (299).

1.4.4.4 Mammalian Sirtuin4.

The human sirtuin4 (SIRT4) gene maps to chromosome 12 (Table 1.7). SIRT4 has been shown to be located within the mitochondrion (Figure 1.10). SIRT4 has NAD-dependent ADP-ribosyltransferase activity and has been reported to downregulate mitochondrial

glutamate dehydrogenase within pancreatic β cells (204). This effect of SIRT4 may be inhibited by CR, perhaps as a result of alterations in the NAD/NADH ratio. The resultant reduction in glutamate dehydrogenase represses glucose and amino acid induced insulin secretion. This activity is in contrast to that of SIRT1, which increases insulin secretion (225). SIRT1-mediated repression of UCP-2 is alleviated by acute starvation and that of SIRT4 on glutamate dehydrogenase is alleviated by chronic CR (204). These may reflect sirtuin regulation of insulin in response to varying types of nutritional stress.

1.4.4.5 Mammalian Sirtuin5.

The human Sirtuin5 (SIRT5) gene maps to chromosome 6 (Table 1.7). SIRT5 has been shown to be located within the mitochondrion (Figure 1.10). SIRT5 has NAD-dependent deacetylase activity. No specific function has yet been attributed to SIRT5.

1.4.4.6 Mammalian Sirtuin6.

The human sirtuin (SIRT6) gene is located on chromosome 19. (Table 1.7). Its encoded protein, SIRT6, has been shown to be located to the nucleus (Figure 1.10), and shows both ADP-ribosyl tranferase and NAD-dependent deacetylase activity (Table 1.7) (7, 205, 206).

1.4.4.6.1 Sirtuin6 and genomic stability.

SIRT6 is a nuclear protein that is associated with heterochromatic regions, including centromeres and telomeres (200). Recently, SIRT6 has been shown to be involved in DNA Base Excision Repair (BER) (300), processes inherent in repairing reactive oxygen species (ROS)-induced DNA damage. SIRT6 was initially thought to be solely a NAD+-dependent ADP-ribosyltransferase (205). However, Mostoslavsky *et al* have demonstrated that SIRT6 can deacetylate histones and DNA polymerase ß (DNA polß) in vitro (300). The latter is a protein involved in BER and is negatively regulated by acetylation via p300 (301). Consequently, SIRT6 has been implicated in DNA repair pathways and the maintenance of genomic stability in cells. Moreover, SIRT6 knockout mice, lacking a normal BER capacity, display numerous progeroid degenerative phenotypes, including lymphopenia, loss of subcutaneous fat and lordokyphosis (300). Telomeric dysfunction, with associated end-to-end chromosomal fusions, might also be a contributory mechanism to this progeroid-like phenotype, as SIRT6 has been shown recently, to also act as a histone H3 lysine deacetylase that modulates telomeric chromatin (206). Although SIRT6-null mice have not been tested under food restriction conditions, the facts that SIRT6-null mice exhibit a severe reduction in circulating IGF-1 levels and that SIRT6 requires its enzymatic

activity to function suggest that it has the potential to serve as a NAD^+ sensor to link nutrient status to genome stability (300).

Recently, SIRT6 has been found to interact with GCIP (106). GCIP is a helix-loop helix leucine zipper protein that functions as a negative regulator of cell proliferation. The expression of GCIP is significantly down-regulated in several human tumours and it has been suggested that it may function as a tumour suppressor gene. Other than acting as a transcription repressor for cyclin D1, it is possible that GCIP could suppress tumourigenesis and promote DNA damage resistance through its interaction with SIRT6 (106).

1.4.4.6.2 Sirtuin6 and NF-κB.

It has been shown that SIRT6 functions at chromatin to attenuate NF-κB signaling. SIRT6 interacts with the NF-κB RELA subunit and deacetylates histone H3 lysine 9 (H3K9) at NF-κB target gene promoters (302).

SIRT6 is recruited to these genomic loci via a physical interaction with RelA, deacetylates histone H3K9, destabilizes RelA from chromatin, and thereby attenuates NF-κB signalling by modifying chromatin at NF-κB target genes. Consistent with these data, in-vivo evidence of increased expression of NF-κB -dependent genes was observed in SIRT6-deficient animals (302).

Since sirtuins are NAD-dependent deacetylases, SIRT6 may act as a nutrient or energy sensor, that links NF-κB signaling to the metabolic state of the organism leading to altered cellular sensitivity to stress induced apoptosis.

1.4.4.6.3 Sirtuin6 and inflammation.

SIRT6 plays a role in inflammation through regulating the production of tumour necrotizing factor (TNF), thus linking the inflammatory response to the metabolic state of the cells (303). Over-expression of wild-type SIRT6, but not the catalytically inactive form, consistently resulted in increased TNF protein production relative to its mRNA. SIRT6 was the only member of the sirtuin family found to positively regulate TNF synthesis (303). The results of this study raised a question concerning the molecular mechanism underlying the observed effects of SIRT6 on TNF mRNA since only nuclear substrates for SIRT6 have been identified to date (206, 300). However, this study demonstrated that optimal TNF translation requires adequate intracellular NAD

concentrations, revealing a potentially crucial link between cellular metabolites and inflammation that might involve SIRT6 (303).

1.4.4.7 Mammalian Sirtuin7.

The human sirtuin7 (SIRT7) gene is located on chromosome 17. No specific enzymatic activity has been described for its encoded protein (127). SIRT7 also displays nucleolar localisation, but specifically associates with nucleolar rDNA (200, 304). SIRT7 interacts with RNA polymerase I (Pol 1) and histones. Overexpression of SIRT7 increases Pol 1-mediated transcription, whereas knockdown of SIRT7, or inhibition of its catalytic activity, results in decreased association of Pol I with rDNA and a reduction of Pol I transcription. These findings suggest that SIRT7 is a positive regulator of Pol 1 transcription, which is required for cell viability in mammals. Decreased SIRT7 activity reduces cell proliferation and triggers apoptosis and increased SIRT7 expression encourages cell growth and prevents apoptosis (Ford et al., 2006).

It was suggested that SIRT7 plays a role in regulating stress response and cell death in the heart through its SIRT7 interaction with p53. SIRT7 can efficiently deacetylate p53 *in vitro* thus decreasing the apoptotic rate in the myocardium (305). This study showed that SIRT7-deficient mice displayed a reduction in mean and maximum lifespan and developed heart hypertrophy and inflammatory cardiomyopathy. SIRT7-deficient primary cardiomyocytes showed an increase in basal apoptosis and in sensitivity to oxidative and genotoxic stress (305).

In contrast to the positive effect of SIRT7 on proliferation, another study suggested an anti-proliferative role for SIRT7 (306). SIRT7 over-expression resulted in slowing-down of the growth of MEF cells and SIRT7 knockout MEFs showed a significant increase in viability rates in both normal and stress conditions (306). The anti-proliferative effect of SIRT7 is also believed to be mediated through p53, but by a distinct mechanism and with a different biological outcome. Previously it was shown that SIRT7 inactivates p53 through deacetylation, thus promoting survival (305). This study demonstrated that SIRT7 is able to activate p53- and c-myc-dependent transcription thus promoting the apoptotic response (306).

Based on the known role of SIRT7 as an activator of rDNA transcription it was proposed that SIRT7 may enable cells to sustain critical metabolic functions by inhibiting cell

growth even under severe stress conditions. Thus SIRT7 expression may improve tissue integrity in aged animals (306).

1.5 Sirtuins and the MTR Trinity.

An interactive trinity controlling the detection of damage associated with ageing and the resultant cellular response has been postulated. The MTR trinity consists of the Mitochondrion, Telomere nucleo-protein complex, and Ribosome biogenesis. The telomere nucleo-protein complexes are part of the DNA damage detection system, the mitochondrion produces energy and is involved in apoptosis, and rDNA controls energy utilization via ribosome production while being in balance with the telomere for protein complex binding (2).

It has been suggested that Sirtuins play an important role in connecting the MTR components in a system that could sense and assess cellular damage and signal the appropriate response in the light of the current redox state (2). This was suggested by the various localisations of sirtuins to the three components of the MTR, and by their NAD-dependent enzymatic activity. Sirtuin activity at the MTR allows the cell to regulate telomeric damage, oxidant load and protein biosynthesis. An imbalance in any one of these components would have a knock-on effect on the others. For example, the accumulation of damage over a cell's lifespan may be associated with accumulating DNA damage and telomere shortening, as well as inefficiency in mitochondrial fuel utilisation, energy production and decreased protein biosynthesis (2). Sirtuin activity at each of these sites may counteract the accumulation of changes associated with the development of biological ageing. Indeed, accelerated ageing is the platform for the development of a number of pathologies, and may be associated with aberrant sirtuin expression at any of these sites (2).

Sirtuins could be important determinants of the sensitivity and extent of the response to DNA damage. Loss of SIRT1 from the telomere results in activation of p53 (231). A further link to ribosomal production may be through p53 activation (controlled in part by SIRT1), as p53 forms a part of a complex interacting with ribosomal protein L5 and 5SrRNA (307). Mitochondrial sirtuins such SIRT3 and SIRT4 , with their NAD-dependent enzymatic activity, may also act as sensors of the redox state of the cell, permitting the cell to regulate metabolism, DNA repair, cell-cycle control and ageing in response to oxidative stimuli (294, 298). SIRT6 may link the DNA damage repair and NF-κB signalling pathway and inflammatory response to the metabolic state of the organism, thus altering the

cellular response to damage and sensitivity to stress induced apoptosis. Finally, SIRT7 controls the transcription of RNA polymerase I, the enzyme that transcribes ribosomal RNA (except for 5S rRNA, which is synthesized by RNA Polymerase III) a type of RNA which accounts for over 50% of the total RNA synthesized in a cell (308). Therefore, sirtuin activity at the MTR allows the cell to regulate telomeric damage, oxidant load and protein biosynthesis. Alteration at any of these components would be reflected in changes at the others. For example, the accumulation of damage over a cell's lifespan may be associated with accumulating DNA damage and telomere shortening, as well as inefficiency in mitochondrial fuel utilisation, energy production and decreased protein biosynthesis.

Sirtuin functions at each of these MTR sites may modulate the response to the changes associated with biological ageing. Indeed, accelerated ageing provides the platform for the development of a number of pathologies, and may be associated with aberrant sirtuin expression at any of these sites (2).

1.6 Sirtuins, ageing and cancer.

As discussed previously (1. 4), accelerated biological ageing is the most potent carcinogen. It is becoming increasingly apparent that molecular and sub-cellular factors considered critical to biological ageing and life span are also factors relevant to neoplastic transformation and tumour growth. There is increasing evidence for the involvement of sirtuins in biological ageing, along with other essential cellular processes including cell cycle control, DNA damage repair and differentiation. However, the involvement of sirtuins in these processes is complex and sometimes apparently contradictory.

1.6.1 Sirtuin1 and ageing.

Several studies showed that SIRT1 negatively regulates cellular senescence, raising the possibility that SIRT1 may promote tumour initiation through its negative effect on cellular senescence. SIRT1 does this by its effect on the expression of tumour suppressors.

The level of SIRT1 protein decreases significantly with serial cell passage in both human lung fibroblasts and mouse embryonic fibroblasts (MFEs) This decrease was inversely correlated with senescence-activated β -galactosidase activity (309). Furthermore, restoration of SIRT1 levels was associated with the spontaneous immortalisation of these cells (309). Consistent with this observation, SIRT1 deacetylates p53 and negatively regulates the cellular senescence response to stress (231).

In contrast, work by various groups raises the possibility that the mammalian SIRT1 may suppress tumour initiation by inducing the expression of tumour suppressors and cellular senescence. Murine SIRT1 was involved in the induction of $p19^{ARF}$ tumour suppressor and cellular senescence induced by chronic oxidative stress (310). This group showed that SIRT1-deficient MEF cells fail to normally up-regulate either the p19 (ARF) senescence regulator, or its downstream target p53. However, following acute DNA damage, or oncogene expression, SIRT1-deficient cells show normal p19 (ARF) induction and cell cycle arrest. Together, these findings demonstrate an unexpected SIRT1 function in promoting replicative senescence in response to chronic cellular stress and implicate p19 (ARF) as a downstream effector in this pathway (310)

1.6.2 Sirtuin1 and cancer.

The role of SIRT1 in tumourigenesis appears to be as complex as its role in ageing. Currently, information concerning the involvement of mammalian sirtuins in tumourigenesis is limited. To date, SIRT1 is not classified as an oncogene, or a tumour suppressor. However, a significant number of studies show that SIRT1 functionally interacts with tumour suppressors and oncogenes. These molecules are either substrates, or interacting proteins, the functions of which are regulated by the enzymatic activity of SIRT1 or proteins that modulate SIRT1 expression. Apparently by acting through these proteins, SIRT1 can have an effect on tumourigenesis.

The cancer disease is a multiple-step process that involves initiation, growth, progression and finally the development of resistance to anti cancer treatments. The function of SIRT1 could vary at different stages of this disease and has to be analyzed in a context specific manner.

SIRT1 plays two different and contradictory roles in controlling cell survival. Both SIRT1 anti-apoptotic and pro-apoptotic effects might be involved in tumourigenesis, and the dominating effect might differ between species and tissue, and could be dictated by the cellular redox state.

1.6.2.1 Sirtuin1 and cancer promoting.

Many studies consistently point to a role for SIRT1 in suppressing cancer cell apoptosis and reducing their sensitivity to agents that cause DNA damage, a role similar to that of yeast Sir2 in response to DNA damage (8, 167, 258). Consistent with the pro-survival effect of SIRT1 (262) found that SIRT1, together with other components of Polycomb Repressive Complex (PRC), a complex that maintain homeotic gene repression during development, are over-expressed in colon, breast and prostate cancers as compared to normal. Another recent study reported that increased SIRT1 expression is associated with a unique subtypes of colon cancer suggesting an involvement of SIRT1 in gene silencing in these subtypes of colon cancer. (311).

1.6.2.1.1 Sirtuin1 and p53 mediated apoptosis.

Various studies showed that p53 activity was negatively regulated by SIRT1-mediated deacetylation (144, 146, 231, 312). Furthermore, SIRT1 deacetylases p73 and negatively controls p73-mediated apoptosis (232).

In contrast to the anti-apoptotic role for SIRT1 shown in mouse MEF cells (144), loss of SIRT1 in both mouse fibroblasts and embryonic stem cells protects against gentoxic stress, in spite of an increase in p53 acetylation (222, 223). Studies with SIRT1-null mice suggest that SIRT1 has little effect on p53-mediated biological outcomes in mice (313, 314). It was also reported that the p53 activity was not altered in response to DNA damage in MEFs engineered so that endogenous p53 could not be acetylated, because six lysine residues were mutated, including lysine 379 (the mouse equivalent of human lysine 382)(315).

1.6.2.1.2 Sirtuin1 and Foxo-mediated apoptosis.

Mammalian Foxo factors are known to cause cancer cell apoptosis, cell cycle arrest and resistance to oxidative stress. Recent studies (316, 317) identified Foxo factors, particularly Foxo1a (316), as tumour suppressors. Foxo factors have also been identified as critical mediators for a stem cell's ability to handle physiological level of oxidative stresses (318).

SIRT1 was shown to deacetylate mammalian Foxo1 and Foxo3a (215, 217, 255) and the deacetylation was consistently found to suppress their pro-apoptotic activity. However, acetylation of Foxo4 by CBP was found to inhibit Foxo4 activity and deacetylation by SIRT1 relieved the inhibition and enhanced the expression of CDK inhibitor, p27Kip (256). Brunet et al (2004) showed that SIRT1 suppressed the induction of pro-apoptotic genes by Foxo3a, but enhanced the induction of genes involved in growth inhibition (e.g. p27 Kip1) and stress responses(215). These studies lead to the suggestion that the deacetylation activity of SIRT1 may suppress Foxo-induced apoptosis, but also induces the activation of cell cycle arresting genes and stress-resistant factors, shifting the balance from apoptosis to stress resistance and survival (215, 319). It is likely that SIRT1 can either lead to activation, or repression, of Foxo-dependent transcription depending on the promoters, cellular context, the external signals, or the proteins that regulate the SIRT1-Foxo interaction (253, 255). For example, the role of SIRT1-mediated deacetylation in oxidative stress induced Foxo1 action in HepG2 141 and 293 138 cells, may differ from Foxo1 action in prostate cancer cells after stimulation with Lysophosphatidic acid (LPA), a potent signalling molecule that stimulates cell proliferation, which was shown to act

through Four and a half LIM domains protein 2 (FHL2), a molecular adaptor linking various signalling pathways to transcriptional regulation (255).

It was found that SIRT1 is over-expressed in human prostate cancer cells when compared with normal prostate epithelial cells and in human prostate cancer tissues compared with adjacent normal prostate tissue (320). Furthermore, its inhibitory actions have anti-proliferative effects in human prostate cancer cells via increasing the acetylation and transcriptional activation of Foxo1(320).

1.6.2.1.3 Sirtuins1 and tumour suppressor genes.

The available data suggest a reciprocal interaction between SIRT1 and tumour suppressor genes (TSGs).

1.6.2.1.3.1 Sirtuin1 and silenced TSGs.

Over-expression of SIRT1 was observed in colon, prostate, breast and lung cancers (262, 314). The over-expression of SIRT1 in colon prostate and lung cancer tumours was correlated with a conserved silencing function of SIRT1 on the promoters of various tumour suppressor genes. SIRT1 localizes to the promoters of aberrantly silenced tumour suppressor genes (321). Furthermore, inhibition of SIRT1 increases the acetylation of histones at endogenous promoters and TSG re-expression despite full retention of promoter DNA hypermethylation (321).

1.6.2.1.3.2 Sirtuin1 and Retinoblastoma protein.

The tumourigenesis promoting effect of SIRT1 is probably due to its direct and indirect negative effects on the biological function of pRb tumour suppressor protein. Wong *et al* demonstrate that SIRT1 can deacetylate pRb tumour suppressor protein *in vitro* and *in vivo*, thus inactivating the growth suppression effect of pRB (272). The indirect effect of SIRT1 on pRb was suggested to be through E2F1. SIRT1 deacetylates E2F1, suppressing its transcriptional activity and proapoptotic function (219).

1.6.2.1.3.3 SIRT1 regulation by TSG.

1.6.2.1.3.3.1 Sirtuin1, p53 and H1C1.

Consistent with the increased level of SIRT1 protein in tumours, the promoter activity of the SIRT1 gene was found to be negatively regulated by tumour suppressors (p53 and DBC1). P53 has been shown to bind to the SIRT1 promoter to repress its expression (247). Under nutritional restriction conditions, Foxo3a is induced which binds and relieves p53-mediated repression, allowing SIRT1 expression to increase. Because the function of p53 is often compromised in tumours, the decreased activity may contribute to the increased expression of SIRT1 in tumour cells. Similar to p53, tumour suppressor HIC1 was found to form a transcriptional repression complex with SIRT1, which directly binds to the SIRT1 promoter and represses its transcription. The loss of HIC1 in tumours results in increased SIRT1 expression and the deacetylation/inactivation of p53, allowing cells to bypass apoptosis and survive DNA damage (249) As discussed earlier, E2F1 increases the expression of SIRT1 by binding to its promoter and directly stimulating SIRT1 transcription, which is thought to be a negative feedback mechanism to suppress E2F1-induced apoptosis (219).

1.6.2.1.3.3.2 Sirtuin1 and Deleted in Breast Cancer 1 (DBC1).

Deleted in Breast Cancer 1 (DBC1) acts as a native inhibitor of SIRT1 activity in human cells and in breast cancer cell lines (250, 322). The deleted in breast cancer 1 (DBC1) gene was identified as a potential tumour suppressor gene that is either found hypermethylated or deleted in cancer (323, 324), and is associated with antiproliferative properties leading to cell cycle arrest in the G1 phase (325). DBC1-mediated inhibition of SIRT1 leads to increasing levels of p53 acetylation and up-regulation of p53-mediated function. Depletion of endogenous DBC1 by RNA interference (RNAi) stimulates SIRT1-mediated deacetylation of p53 and inhibits p53-dependent apoptosis (250, 322).

1.6.2.1.4 Ku70 deacetylation and the suppression of Bax release into mitochondria by Sirtuin1.

SIRT1 deacetylates Ku70 at K539 and K542 sites. Such an action strengthens the ability of Ku70 to sequester Bax away from mitochondria and inhibited cell death mediated by Bax. Bax is a pro-apoptotic member of the Bcl-2 family whose localization to mitochondria triggers cytochrome c release and apoptosis, as described in section 1.5.4.1.2.4.

1.6.2.1.5 Cytoplasmic action of SIRT1.

Although, the main prosurvival function of SIRT1 is attributed to its nuclear action, a recent study (266) showed that SIRT1 is cleaved by caspase during the apoptosis of N2a neuroblastoma cells, which is associated with increased cytoplasmic localization of SIRT1. The data suggest the possible involvement of cytoplasmic action of SIRT1 in cell death. The studies, however, did not make it clear whether the cleavage and increased cytoplasmic localization is a consequence of cell apoptosis or a cause. It is also unclear whether the cleavage product still functions as a NAD+-dependent deacetylase.

1.6.2.2 Anticancer effects of sirtuin1.

Several studies suggest that SIRT1 may act as a tumour suppressor. MEFs derived from SIRT1-null mice are prone to spontaneous immortalization, suggesting that SIRT1 behaves as a growth-suppressive gene in culture (310). Furthermore, hematopoietic stem cells from SIRT1-null mice have increased proliferation potential, and shRNA knockdown of SIRT1 in human fibroblasts accelerates cell proliferation (230, 326). Transgenic over-expression of SIRT1 in the intestine inhibited polyp formation in the ApcMin mice (327), whereas SIRT1 deficiency led to increased tumour formation in p53-null mice(328). SIRT1 has also been shown to inhibit androgen receptor dependent cell proliferation in prostate tumour cells (277). These observations suggest that SIRT1 may suppress tumour growth under certain conditions, and that SIRT1 activators could be used for cancer treatment or prevention (329).

1.6.2.2.1 Sirtuin1 and NF-κB.

An anti-cancer effect has been observed for SIRT1, through its interaction with NF-κB, an important regulator of ageing-related cellular processes. Over-expression of SIRT1 in 293 cells and small cell lung carcinoma cells sensitized the cells to apoptosis induction by TNFα (218). In breast cancer SIRT1 is recruited to suppress the activity of NFκB by neddylated breast cancer associated protein 3 (BCA-3) (261).

Given the known association between inflammation and tumourigenesis, a role for SIRT1 in suppressing inflammation and tumourigenesis of the lungs has been suggested. Yang et al (2007) showed that cigarette smoke extracts decreases SIRT1 activity, consequently increasing NFκB-dependent release of pro-inflammatory mediators.

1.6.2.2.2 Sirtuin1, Retinoblastoma protein, and E2F1.

Another anti-cancer effect for SIRT1 was observed in colon cancer. It was reported that SIRT1 levels were increased in normal colon mucosa and benign adenomas and decreased in approximately 30% of carcinomas. Furthermore, pharmacological inhibition of SIRT1 stimulates cell proliferation under conditions of growth factor deprivation. Consistent with this effect, it has been found that found that elimination of SIRT1 accelerates tumour xenograft formation by HCT116 cells, whereas SIRT1 over expression inhibits tumour formation (330). Paradoxically, SIRT1 inhibition also sensitizes cells to apoptosis by chemotherapy drugs (330). This study suggested that pRb hyper-phosphorylation (inactive form), as an indirect result of SIRT1 inhibition, is probably responsible for this tumourigenic effect of decreased SIRT1. Decrease SIRT1 activity results in activation of E2F1 which can promote pRb hyper-phosphorylation indirectly through induction of cyclinD/cdk4 activity thus promoting pRb phosphorylation.

This suggested pathway adds to the intricacy of the role of SIRT1 in tumourigenesis, as it apparently contradicts the previously mentioned effect of SIRT1 on pRb (219, 272). It has been reported that SIRT1 might promote cancer directly, through deacetylation of pRb that results in enhancing pRb phosphorylation (inactivation) (272), and indirectly, through deacetylation, and suppression of the E2F1 transcriptional activity and proapoptotic function (219).

1.6.2.3 SIRT1 and cancer treatment.

The involvement of sirtuins in the cancer disease process suggest that they are potential novel molecular targets for anticancer treatment. Consistent with the increased level of SIRT1 protein in tumours, SIRT1 inhibitors have been shown to exhibit anti-tumour activity (331). Treatment of BCL6-expressing lymphoma cells with cambinol, an inhibitor of the enzymatic activity of SIRT1 and SIRT2, induces apoptosis (331). Moreover, sirtinol induces senescence-like growth arrest in human breast cancer MCF-7 cells and lung cancer H1299 cells (332). Consistent with the cancer cell specific prosurvival function, SIRT1 expression also increases the resistance of cancer cells to therapeutic treatments with ionizing radiation (146), etoposide (219) or cisplatin treatment (333). In addition, the expression of SIRT1 was found to be increased both at the RNA and protein levels in five drug-resistant cell lines when compared to their drug-sensitive counterparts (334). Biopsies from cancer patients treated with chemotherapeutic agents also expressed high levels of SIRT1 but not other sirtuins.

Furthermore, the interaction between SIRT1 and E2F1 suggests an involvement of SIRT1 in resistance to chemotherapy. SIRT1 deacetylates E2F1, and suppresses its transcriptional activity and pro-apoptotic function (219). Interestingly, there is a negative feedback loop between E2F1 and SIRT1, as E2F1 induces SIRT1 expression, and SIRT1 binds to and inhibits E2F1 activity (219). Inhibition of SIRT1 results in an increase in E2F1-mediated apoptosis. SIRT1 may prevent E2F1-mediated apoptosis, thereby allowing DNA damage repair.

Finally, inhibition of SIRT1 reactivates the silenced tumour suppressor genes without loss of promoter DNA hypermethylation. This was consistent with the silencing effect of SIRT1 on TSGs (321). Recently, it has been reported that Salermide, a strong *in vitro* inhibitor of SIRT1 and SIRT2, has a strong cancer-specific proapoptotic effect. The pro-apoptotic effect of Salermide is probably due to reactivation of proapoptotic genes repressed exclusively in cancer cells by SIRT (335). SIRT1 inhibition also sensitizes cells to apoptosis by chemotherapy drugs (330).

1.6.2.3.1 Sirtuin1 and resistance to chemotherapy.

Finally, a role for sirtuins was suggested in developing resistance to chemotherapy. Eliminating SIRT1 using the siRNA significantly reversed the resistance phenotype and reduced expression of the multidrug resistance molecule P-glycoprotein. Ectopic expression of SIRT1 induced expression of P-glycoprotein, and rendered cells resistant to doxorubicin. Collectively, these studies suggest that SIRT1 is a molecular target to reverse the resistance of cancers to chemotherapy (334).

1.6.3 Sirtuin2, cancer progression and invasion.

SIRT2 has a negative effect on tumour progression through its interaction with microtubules that are involved in mitosis and cell cycle control. SIRT2 deacetylates the α-tubulin subunit of microtubules and delayed the progression of cell cycle and participates in a late mitotic checkpoint to ensure correct chromosome segregation during cytokinesis (201, 286, 336). Consistent with these observations, SIRT2 has been found to be down regulated in gliomas (336) and its ectopic expression suppresses the growth of glioma cell lines. Therefore, decrease in SIRT2 activity might assist DNA-damaged tumour cells in escaping cell cycle arrest.

Similarly, since SIRT2 could control the cytoskeleton, a putative negative effect of SIRT2 on tumour invasion and metastasis could be suggested.

1.6.4 Sirtuin3, Sirtuin4, Sirtuin5 and cancer.

Although little is known about the functions and biological roles of SIRT3, SIRT4 or SIRT5, their conserved NAD-dependent enzymatic activity and their interesting mitochondrial localisation is suggestive of similar involvement in cell responses to various kinds of stress, in cell cycle progression and/or DNA repair. Similarly, it is reasonable to assume that these sirtuins will play a role in cancer occurrence and/or progression.

Recently, the availability of more information regarding SIRT3 is supportive of its potential role in cancer disease. It was shown that SIRT3 has a pro-apoptotic function and might be implicated in the apoptotic mitochondrial pathway (296). Furthermore, the role of SIRT3 in cancer might be even more complicate by the recent study identifying SIRT3 as a novel SIRT1 protein interacting partners (299).

1.6.5 *Sirtuin6 and cancer.*

SIRT6 deacetylates and activates DNA polymerase ß (a protein involved in BER). Thus SIRT6 promotes normal DNA repair and the loss of SIRT6 leads to abnormalities in mice that overlaps with ageing-associated degenerative processes (300). SIRT6 also act as a histone H3 lysine deacetylase that modulates telomeric chromatin structure, promoting telomeric stability (206). Therefore, an anti-cancer role could be suggested, as loss of SIRT6 might predispose to cancer in the presence of cellular stress.

SIRT6 might also be involved in preventing tumour progression. The anti cancer role for SIRT6 could be mediated through its negative effect on cellular proliferation resulting from:

SIRT6 interaction with the NF-κB RELA subunit and deacetylates histone H3 lysine 9 (H3K9) at NF-κB target gene promoters, consequently attenuating NF-κB signalling (302). This could increase cellular sensitivity to stress-induced apoptosis.

SIRT6 interaction with GCIP (a negative regulator of proliferation) (106). The expression of GCIP is significantly downregulated in several human tumours and it has been suggested that it may function as a tumour suppressor gene. GCIP could suppress

tumourigenesis and promote DNA damage resistance through its interaction with SIRT6 thus reducing the chance of developing genomic instability that might leads to cancer (106). Decreased expression of SIRT6 in breast cancer could therefore be an alternative and/or synergistic mechanism for cells to overcome the tumour suppressor effect of GCIP.

Another possible involvement of SIRT6 in cancer disease could be related to it role inflammation. Recently, a role for SIRT6 in inflammation has been reported through regulating the production of TNF (303) and suggesting link between metabolism and inflammatory response. Although, it could be limited to some disease such as ulcerative colitis and gastritis the association between inflammation and tumorigenesis is well known. It has been shown that the intense of systemic inflammatory response (SIR) (indicated by modified Glasgow prognostic score (mGPS) that associated with cancer disease has a prognostic value. mGPS is calculated as follows: patients with elevated C-reactive protein (CRP) serum levels (>10mg/L) and hypoalbuminaemia (<35g/L) were allocated a score of 2, patients with elevated CRP serum levels without hypoalbuminaemia were allocated a score of 1, patients with normal CRP serum levels with or without hypoalbuminaemia were allocated a score of 0. The prognostic value for the mGPS has been proven for different cancers such as colon, rectal, gastroesophageal, breast and lung cancers (337-341). The involvement of SIRT6 in inflammation could indicate a potential role for SIRT6 in modulating SIR and suggest a possibility of novel treatments targeting SIRT6 activity in order to dampen the SIR.

1.6.6 Sirtuin7 and cancer.

SIRT7 involvements in cancer disease is not simple in as SIRT7 has been shown to have both positive and negative effect on cellular proliferation.

SIRT7 might promote cancer through its positive effect on the transcription of RNA polymerase I (Pol I). Decreased SIRT7 activity reduces cell proliferation and triggers apoptosis, while increased SIRT7 expression encourages cell growth and prevents apoptosis (Ford et al., 2006). Consistent with these observations, SIRT7 expression was reported to be increased in both thyroid cancer cell lines and biopsies (342). Therefore a role in the progression of cancer could be suggested for SIRT7.

On contrary, a negative effect for SIRT7 on cancer was suggested depending on the antiproliferation effect of SIRT7 (306). This study showed that increased SIRT7 is able to activate p53- and c-myc-dependent transcription thus promoting apoptosis (306).

Furthermore, decreased expression of SIRT7 was observed in murine tumourigenic cell lines: P19 (teratocarcinoma), NB41A3 (neuroblastoma), C3H/MCA (transformed fibroblast-derived cell line) as compared to the control non-tumourigenic cell line C3H/10T1/2 (306). Therefore, it has been suggested that decreased SIRT7 levels promote the cancer disease process.

1.7 Aims.

Breast cancer is one of the most common cancers to affect women, with over 40,000 new cases being detected and around 13,000 women dying from the disease in Britain each year. Unsurprisingly, the study of breast cancer biology is a major area in cancer research. Intensive efforts to define the molecular events that lead to breast cancer and to correlate these events with its clinical behaviour have been made in recent years. It is becoming increasingly apparent that molecular and sub-cellular factors considered critical to cellular senescence and organismal life span are also factors relevant to neoplastic transformation and tumour growth. Given the predicted effects of sirtuins on ageing, we hypothesise that aberrant sirtuin activity might be involved in the cancer disease process. Sirtuins might contribute both to the pathogenesis of cancer and to the response of tissue to anti-tumour agents.

To test this hypothesis we decided to compare the relative transcriptional levels of sirtuin genes in breast cancer disease with a view to trying to associate the level of transcriptional expression with clinico-pathological features of the disease. Furthermore, we sought to investigate the relative transcriptional expression levels of sirtuin genes in breast cancer cell lines in response to different anti-tumour treatments. Consequently, the study has been divided into two main parts:

A. An *In Vivo* study that aims to:

1. Investigate the relative transcriptional expression levels of Sirtuin genes in three groups of breast biopsies:

i. Normal (from breast reduction).

ii. Non-malignant (normal tissue from mastectomy).

iii. Malignant (malignant tissue from mastectomy).

B. An *In Vitro* study that aims to:

1. Investigate the relative transcriptional expression levels of Sirtuin genes in response to adjuvant chemotherapy used in breast cancer:

i. Investigate the relative transcriptional expression levels of Sirtuin genes in response to Tamoxifen in :

1. ER positive breast cancer cell line (MCF-7).

2. ER Negative breast cancer cell line (MDA-MB-453).

ii. Investigate the relative transcriptional expression levels of Sirtuin genes in response to Docetaxel in:

1. ER Negative breast cancer cell line (MDA-MB-231).

Chapter II

Materials and Methods

2.1 In vivo study (gene expression in breast biopsies)

2.1.1 Breast biopsies

Gene expression was studied in three groups of breast samples. These comprised normal breast biopsies (n = 26), non-malignant (n = 78) and malignant (n = 73) breast biopsies. Normal breast tissue samples were obtained from patients who had undergone breast reduction surgery (reduction mammoplasty) at the Golden Jubilee national hospital between 2006 and 2007. The tissue samples were stored in RNA-later (Ambion, UK) at -20^0C. Non-malignant and malignant breast tissue samples were obtained from mastectomy samples following routine histopathological analysis that confirmed the presence or absence of malignancy in the biopsy. These operations were carried out for potentially curable invasive ductal carcinoma in the period between 1987 and 2000. Portions of tissue specimens not required for diagnosis, or staging, were snap frozen and stored in liquid nitrogen at Department of Surgery Western Infirmary, Glasgow. Prior permission for storage and future analysis of tissue samples was obtained from all patients. Ethical approval for this study was obtained from the local ethics committee, to use anonymous specimens, banked in this manner, for research purpose and to correlate these research findings with additional clinical parameters and outcomes, using the hospital code number.

2.1.1.1 Tissue processing:

Breast glandular tissue was carefully dissected free from surrounding fatty tissue. Then frozen tissues were disrupted under liquid nitrogen, using a mortar and pestle, until a powder was obtained. The TRIzol method of total RNA extraction was used; briefly, 1ml of TRIzol reagent was added per 50-100 mg of initial breast tissue and homogenised, using a series of decreasing gauge needles and a syringe (if required). This was then incubated for 10 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 mls of chloroform was then added for each 1 ml of TRIzol reagent used for the initial homogenisation. The tubes were left at room temperature for 3 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. The resultant aqueous phase was carefully removed and transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol; 0.5 ml of isopropyl alcohol was added per 1ml of TRIzol reagent used for the initial homogenisation. The tubes were mixed and incubated at room temperature for 10 minutes, to allow RNA to precipitate before being

centrifuged at 12,000g, for 10 minutes at 4°C, to pellet the RNA. The supernatant was removed and the RNA pellet washed by adding 1ml of 75% ethanol, vortexing and centrifuging at 7,500g, for 5mins at 4°C. The RNA pellet was air-dried for 10 minutes and resuspended in nuclease-free water. Samples were incubated at 57.5°C for 10 minutes to enhance dissolution before being chilled on ice for 5 minutes.

The RNA concentration and purity were determined by measuring absorbance of the sample at 260nm, using a GeneQuant capillary spectrophotometer (Pharmacia Biotech). Samples were stored at –80°C.

Initial qualitative assessment of the RNA was tested by running 500ng of each sample on a 1% (non-denaturing) agarose gel in TBE (Tris Borate Ethylenediaminetetracetic acid EDTA) containing 0.5μg/ml Ethidium Bromide and visualising under UV light (Figure 2.1).

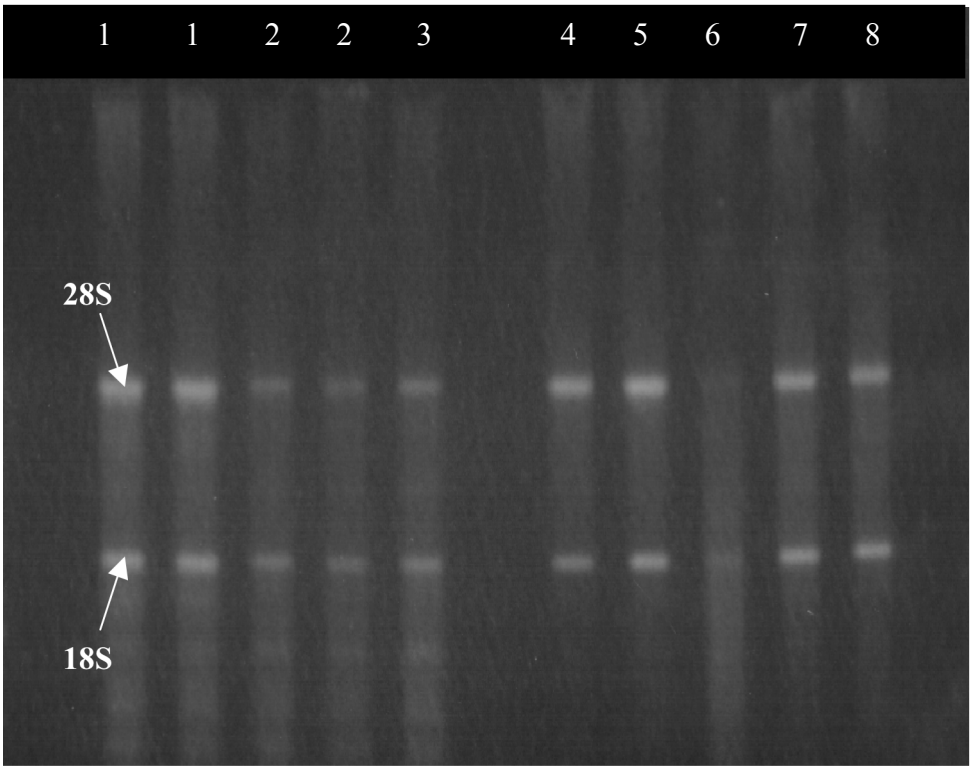

Figure 2.1: Gel analysis of RNA extracted from breast cancer tissue.

Arrows indicate the two bands that represent the ribosomal RNA (18S and 28S). Samples with degraded RNA (6), indicated by the replacement of the two clear bands with unspecific smear on the gel, were excluded from further analysis.

2.1.1.2 DNase treatment:

DNase treatment was performed using the commercially available DNA-Free Kit (Ambion, UK). 20 μL of RNA was added to a tube containing 2-3 units of DNase I and 0.1 volume of 10× DNase I Reaction Buffer. The contents of the tube were mixed gently and

incubated for 30 minutes at 37°C. The DNase I was then inactivated by adding 0.1 volume of DNase Inactivation Reagent. The mixture was incubated for 2 minutes at room temperature, and then centrifuged for 1 minute to pellet the DNase Inactivation Reagent. The supernatant, which contains the DNase-treated RNA, was transferred to a new 0.5ml eppendorf tube and stored at –80°C.

2.1.1.3 cDNA Synthesis:

Two micrograms of DNase treated RNA was then reverse transcribed into cDNA using the Superscript First-strand Synthesis System (Invitrogen, UK) for reverse transcriptase PCR following the manufacturer's recommendations. This part of the study was performed in duplicate with each sample being treated with and without the reverse transcriptase (RT) enzyme as a control. Random hexamer based transcription was performed for the breast biopsy studies and oligo dT based transcription for experiments using mammary cell lines. Random hexamer-based transcription has been shown to generate a more normalised cDNA pool from sources that may contain partially degraded RNA (Schwabe, Stein et al. 2000).

2.1.1.3.1 Hexamer-based RT reaction:

1 µl of 10 mM dNTP mix and 2 µl hexamers were added for every 2 µg of RNA to be reverse transcribed. The total volume was made up to 12 µl using Nuclease-free water. The mixture was then incubated at 65°C for 5 minutes before being placed on ice. 4 µl of 5X RT Buffer, 2 µl of 0.1M DTT and finally 1 µl of the RNase inhibitor (Rnase OUT) were then added, bringing the total volume up to 19µl. This mixture was gently mixed then briefly centrifuged before being incubated at 20°C for 2 minutes. 1µl of the reverse transcriptase enzyme, SuperScript II, was then added, but only to those samples to be reverse transcribed and not to the negative controls (–RT). The total mixture was then incubated at 25°C for 10 minutes, at 42°C for 50 minutes, terminated at 75°C for 15 minutes and finally chilled on ice for 5 minutes. Finally, the reactants were treated with 1 µl of RNase at 37°C for 20 minutes. Nuclease-free water was then added to obtain a final concentration of 100 ng per 8 µl. The specimens were then stored at -20°C.

2.1.1.3.2 Oligo dT-based RT reaction:

1 µl of 10 mM dNTP mix and 1 µl oligo dT was added for every 2 µg of RNA to be reverse transcribed. The total volume was made up to 12µl using nuclease-free water. The

mixture was then incubated at 60°C for 5 minutes before being placed on ice. 4 μl of 5X RT Buffer, 2 μl of 0.1M DTT and finally 1μl of the RNase inhibitor, RnaseOUT, were then added, bringing the total volume up to 19μl. This mixture was gently mixed then briefly centrifuged before being incubated at 42°C. 1μl of the reverse transcriptase enzyme, SuperScript II, was then added, but only to those samples to be reverse transcribed and not to the negative controls (–RT). The total mixture was then incubated at 42°C for 50 minutes, terminated at 75°C for 15 minutes and finally chilled on ice for 5 minutes. Finally, the reactants were treated with 1μl of RNase at 37°C for 20 minutes. Nuclease-free water was added to obtain a final concentration of 100ng per 8 μl. The specimens were then stored at -20°C.

2.1.1.4 Reverse Transcriptase-Polymerase Chain Reaction and Template validation.

A basic Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis was used to assess the utility of the cDNA templates using a human β-actin control primer set that has been designed using Primer Express (Table 2.1).

Table 2.1: Forward and reverse primer sequences used for human β-actin Taqman.

Gene	Forward Primer	Reverse primer
β-actin	GGTCACCCACACTGTGCCCAT	GGATGCCACAGGACTCCATGC

The RT-PCR for β-actin was performed on the breast cDNA samples using the DNA Engine (PTC-200, Bio–Rad) in a 50 μl reaction in 0.2 ml PCR tube.

The mixture consisted of 12.5ng of cDNA, 1μl of 20mM forward and reverse primers (final concentration of 400 nM), 10 μl of 10X PCR buffer minus Mg, 1μl of 10 mM dNTP, 1.5 μl of 50 mM Mg Cl2, 0.25 μl of Taq DNA polymerase (5U/μl), and Nuclease-free water to make up the reaction volume to 50 μl.

Cycle conditions that represent the Basic PCR protocol were followed:

- Denature: 94°C for 3 minutes.
- 30 cycles of PCR amplification as follow
  - *Denature: 94°C for 45 seconds.*
  - *Anneal: 61°C for 30 seconds.*
  - *Extend: 72°C for 1 minute and 30 seconds.*

- Final extension: 72 °C for 10 minutes.

The reaction was then maintained at 4°C until being analysed using gel electrophoresis or stored at -20°C.

The reaction products were analysed by gel electrophoresis. An intact band at the expected size of 350 bp indicated RNA of usable quality that could be processed further for gene quantification using the Taqman PCR (Figure 2. 2). Only cDNA samples that produced an intact β-actin band were selected for subsequent gene expression studies.

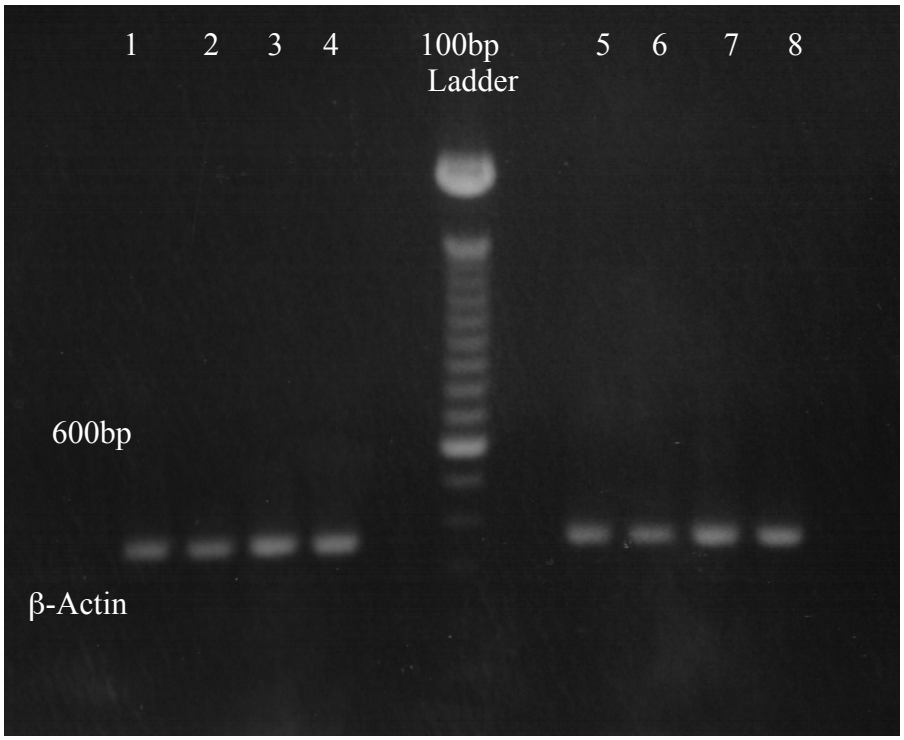

Figure 2.2: Gel analysis of the RT-PCR products for β-Actin.

The cDNA used was produced from RNA extracted from different breast cancer tissues. (Lanes 1 to 8) All samples showed a band at the expected size of 350 bp.

2.1.2 Relative Quantitative Real Time PCR (Taqman).

Relative Quantitative Real time PCR was used for cDNA quantitation analysis in order to monitor the mRNA expression patterns of the genes of interest. The analysis was performed using the ABI Prism® 7700 and 7900 Sequence Detection System.

2.1.2.1 Basics of the Assay.

The Real time PCR reaction exploits the 5' nuclease activity of AmpliTaq Gold TM DNA polymerase to cleave a non-extendable oligonucleotide hybridisation probe (Taqman® probe) during the PCR reaction. The probe is labelled with a reporter fluorescein dye, 6-

carboxy-fluorescein (FAM), at the 5' end and a quencher fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA), at the 3' end. When the probe is intact, the reporter dye emission is quenched due to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, the nucleolytic activity of the Taq DNA polymerase cleaves the hybridisation probe and releases the reporter dye from the probe (Figure 2. 3). With each cycle of PCR amplification there is an increase in fluorescence emission of the reporter which is monitored in real-time using the ABI Prism 7700 sequence detector (Heid, Stevens et al. 1996). The 3' end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. Briefly, accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Since increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR, any non-specific amplification is not detected.

The sequence detector is a combination of a thermal cycler, laser and Sequence Detecting system software (SDS) that automates 5' nuclease-based detection and quantitation of nucleic acid sequences. Using the Taqman Universal PCR mastermix provides an internal reference to which the reporter dye signal is normalised during data analysis as it contains a passive reference dye (ROX). Normalisation is necessary in order to correct for fluorescent fluctuations due to changes in concentration or volume, and is achieved by dividing the emission intensity of the reporter dye by the emission intensity of the passive reference dye to obtain a ratio defined as the Rn (normalised reporter) for a given reaction tube. Rn+ is the Rn value of a reaction containing all components including the template. Rn- is the Rn value of an untreated sample. This value may be obtained from the early cycles of a Real Time run, prior to a detectable increase in fluorescence or from a reaction not containing template. Then a computer algorithm compares the two values of Rn generating a ΔRn value. The ΔRn value indicates the magnitude of the signal generated by the given set of PCR conditions and reflects the amount of hybridised probe that has been degraded.

The following equation expresses the relationship of these terms:

$$\Delta Rn = (Rn+) - (Rn-)$$

$$Rn+ = \frac{\text{Emission intensity of reporter}}{\text{Emission intensity of passive reference}} \quad \text{(PCR with template)}$$

Rn- = Emission intensity of reporter (PCR without template)

Emission intensity of passive reference

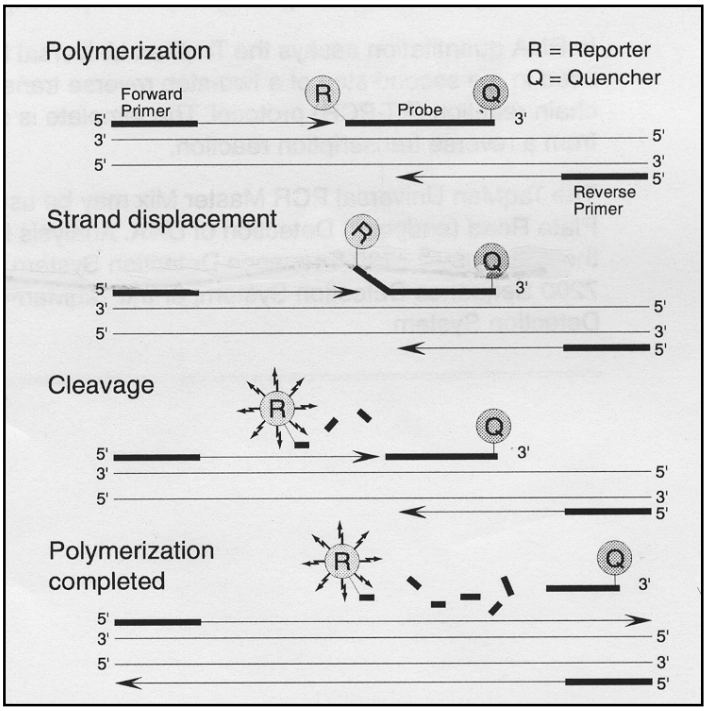

Figure 2.3: Schematic representation of the steps involved in the real-time Taqman PCR reaction.

First the primers and probe anneal to the cDNA transcript. There is no fluorescence because the reporter dye emission is quenched. Then the primers are extended during the extension phase of the PCR cycle, during which the 5'-3' exonuclease activity of the AmpliTaq Gold DNA Polymerase cleaves the hybridised probe and releases the reporter dye resulting in an increase in reporter fluorescent dye emission. Finally the primers continue to be extended until polymerisation of the amplicon is complete [Illustration adapted from Applied Biosystems Taqman Manual].

2.1.2.1.1 Taqman data parameters.

The Sequence Detection System (SDS) creates quantifiable relationships between test samples based on the number of cycles elapsed before achieving detectable levels of fluorescence. For this to be achieved two parameters, Threshold and baseline, have to be set.

The baseline: is the noise level in early cycles, typically measured between cycles 3 and 15, where there is no detectable increase in fluorescence due to amplification products(Figure 2.4).

The Threshold: is adjusted to a value above the background and significantly below the plateau of an amplification plot. It must be placed within the linear region of the amplification, which is automatically determined by the computer software, and defined as the average standard deviation of Rn from a predefined range of PCR cycles (baseline), multiplied by an adjustable factor (Figure 2.4). The threshold can be adjusted manually provided that it lies in the linear phase of the amplification plot where the Rn value doubles every cycle.

The threshold cycle (Ct): The threshold cycle (Ct) for a given amplification curve is the cycle number at which the fluorescence generated within a reaction well exceeds the defined threshold and indicates a point at which a significant increase in ΔRn is first detected (Figure 2.4). The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number of the target nucleic acid.

2.1.2.1.2 Endogenous reference gene.

This is a gene whose expression level should not differ between samples, such as housekeeping genes. Amplification of an endogenous control/ reference gene (housekeeping) is vital in order to standardise the amount of cDNA added to each reaction. The level of expression of the housekeeping gene should be similar to that of the genes being investigated. Initially, two housekeeping genes 18S and Hypoxanthine Ribosyl Transferase (HPRT) were tested for this study. Validation experiments showed that HPRT is a better housekeeping gene for determining the relative levels of Sirtuin gene expression. Therefore, for this study the internal control gene used was Hypoxanthine Ribosyl Transferase (HPRT). Taqman PCR was performed using primer/probe sets simultaneously for both genes of interest and the housekeeping genes.

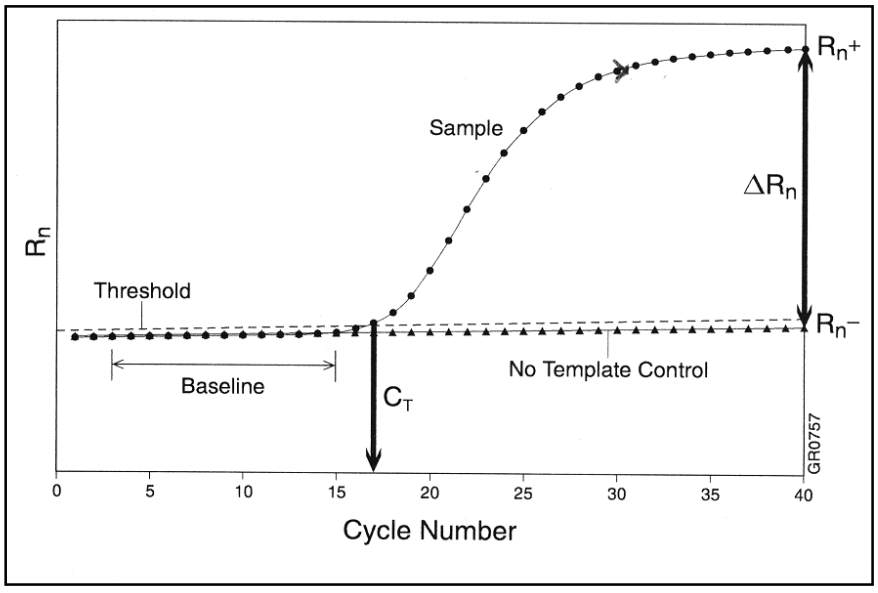

Figure 2.4 Schematic

representation of a real time PCR amplification.

The threshold cycle occurs when the sequence detection application begins to detect the increase in signal associated with an exponential growth of PCR product. (illustration adopted from PE Applied Biosystems Taqman Manual).

2.1.2.1.3 Relative gene expression calculation.

The threshold cycle number (Ct) for both gene of interest and housekeeping gene was recorded for each sample. This allowed the expression of the gene of interest to be normalized to the endogenous housekeeping gene. This was performed by calculating the ΔCt_{Sample} by subtracting the Ct value of the endogenous housekeeping gene from the Ct vale of the gene of interest ($\Delta Ct_{Sample} = Ct_{Gene\ of\ interest} - Ct_{Housekeeping\ gene}$).

The comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method (399), was used for calculating the relative gene expression. This involved comparing the ΔCt values of the samples with a control or calibrator, such as cDNA from a non-treated sample or normal tissue. This involves calculating the ΔΔCt, by subtracting $\Delta Ct_{Calibrator}$ from the ΔCt_{Sample}.

2.1.2.2 Taqman probe and primer design

Taqman primer and probe sequences for SIRT1, SIRT2, SIRT3, SIRT7(Appendix 1), p21, p16, XRCC5, 18S and HPRT genes were designed de novo from sequences in the Genebank database using Primer Express software The sequences of the primers and probe for each of these genes are presented in (Table 2.2). All primer/probe sets were designed such that at least one of the oligonucleotides crossed an intron/exon boundary. The Taqman primers and the FAM-TAMRA-conjugated probe were designed using the Primer Express software. The software generates a list of potential probe and primer candidates based on default values of melting temperatures (Tms) and GC content.

However, for successful amplifications the following requirements must be met manually:

- The probe sequence should contain more Cs than Gs and should not have a G on the 5' end.

- Both the forward and reverse primers should not have more than two C+Gs within the last five bases of the 3' end.

All primer/probe sets were subsequently tested by BLAST searching and *in silico* amplifying the cDNA. This was to ensure that the primers and probe did not bind other sequences and that primer dimers were not formed.

Taqman primers and probes for SIRT4, SIRT5, SIRT6, BCL2, and Ki67 were purchased as pre-designed and validated primers and probes from Applied Biosystems UK (Table 2.3).

Table 2.2: A list of the primer and probe sequences used for Taqman analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Taqman™ Probe (5' FAM – TAMRA 3')
SIRT1	TAGAGCCTCACATGCAAGCTCTA	GCCAATCATAAGATGTTGCTGAAC	ACTCCAAGGCCACGGATAGGTCCATATACTT
SIRT2	CCTCGCCTGCTCATCAACA	TCCTCCGAGGCCCATAATC	TGGCCAGTCGGACCCTTTCCTG
SIRT3	CATTCGGGCTGACGTGATG	AACCACATGCAGCAAGAACCT	TGCACCGGCGTTGTGAAGCC
SIRT7	CGTCCGGAACGCCAAATAC	GACGCTGCCGTGCTGATT	TGGTCGTCTACACAGGC
p21	GCAGACCAGCATGACAGATTTCTA	GCGGATTAGGGCTTCCTCTT	CACTCCAAACGCCGGCTGATCTTC
p16	CATAGATGCCGCGGAAGT	CCCGAGGTTTCTCAGAGCCT	CCTCAGACATCCCCGATTGAAAGAACC
HPRT	CTTGCTCGAGATGTGATGAAGG	CAGCAGGTCAGCAAAGAATTTATAG	ATCACATTGTAGCCCTCTGTGTGCTCAAGG
XRCC5	TTGATTTGCTGGAGGACATTGA	TCCATGCTCACGATTAGTGCAT	CAACCAGGTTCTCAACAGGCTGACTTCC
18 S	ACCTGGTTGATCCTGCCAGTAG	AGCCATTCGCAGTTTCACTGTAC	TCAAAGATTAAGCCATGCATGTCTAAGTACGCAC

Table 2.3: A list of pre designed and validated primers and probes and their manufacturer's code purchased form Applied biosystems.

Gene	Manufacturer's code
SIRT4	HS00202033_m1
SIRT5	HS00229729_m1
SIRT6	HS00213036_m1
KI67	HS00606991_m1
BCL2	HS00153350_m1

2.1.2.3 Primer and probe concentration optimisation for real time PCR.

The primer and probe concentration need to be optimised in order to achieve a satisfactory amplification. The purpose of this optimisation experiment is to determine the minimum

primer/probe concentrations that produce the maximum fluorescent signal and the smallest Ct number. For that purpose Taqman real time PCR reactions were initially performed with 50 ng cDNA template, 200nM probe concentration, and different combinations of forward (Fwd) and reverse (Rev) primer concentrations as shown on the (Table 2. 4):

Table 2.4: Combinations of forward and reverse primer final concentrations used in probe, primer concentration optimisation experiments for Real time PCR.

50 nM fwd / 50 nM rev	50 nM fwd / 300 nM rev	50 nM fwd / 900 nM rev
300 nM fwd / 50 nM rev	300 nM fwd / 300 nM rev	300 nM fwd / 900 nM rev
900 nM fwd / 50 nM rev	900 nM fwd / 300 nM rev	900 nM fwd / 900 nM rev

This experiment was performed for SIRT1, SIRT2, SIRT3, SIRT7 only as SIRT4, SIRT5, SIRT6, KI67 and BCL2 had been already optimised by the company and their final concentration in the PCR reaction is 900 nM for both primers and 225 nM for probe per well. This experiment indicated that the optimal combination of primer concentrations for the quantitative amplification of SIRT1, SIRT2 and SIRT3 was 900 nM fwd/ 900 nM rev. However, the combination of 300 nM fwd / 900 nM rev proved to be most efficient for amplifying SIRT7 using the real time PCR.

The combination of primer concentrations of (300 nM fwd / 300 nM rev) was used for quantitative amplification of p16, p21, and XRCC5. This has been adopted from previous optimisation experiments performed in our laboratory.

Using the pre-determined optimal primer concentrations, another experiment was performed to determine the optimal probe concentration using the following concentrations: 125 nM, 150 nM, 175nM, 200 nM, 225 nM, and 250 nM. The result of this experiment indicated that a probe concentration of 225 nM was the optimal concentration for SIRT1, SIRT2, SIRT3, and SIRT 7. A probe concentration of 200 nM was used for p16, p21, and XRCC5 as recommended from previous experimental results performed in our laboratory.

2.1.2.4 Taqman cDNA amplification reaction

Relative Quantitative Real time PCR was performed in 96-well plates using a 25 µl reaction. The mixture was prepared in triplicate according to the following recipe:

12.5 µl of 2X Taqman Universal PCR Mastermix

1 µl 5 -5.62µM probe	Final concentration of (200- 225 nM)
1.5 µl 5-15 µM Forward primer	Final concentration of (300 -900 nM)
1.5 µl 5-15 µM Reverse primer	Final concentration of (300- 900 nM)
8 µl cDNA	Final concentration of (25-50 ng)

H_20 (Nuclease free water) to 25 µl

The concentration of primers and probe were used according to the optimisation experiment (see above).

1.25µl of primer and probes mix was used for quantitative amplification of SIRT4, SIRT5, SIRT6 and BCL2, and Ki67 according to the manufacturer's instructions. This produces final concentrations of 900 nM fwd / 900 nM rev for the primers and 225 nM for the probe.

Cycle conditions that represent the Basic Real time PCR protocol were 50°C for 2 minutes, followed by 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute.

No Amplification Control (NAC) or No Template Control (NTC) were always included in each real time PCR run.

2.1.2.5 Primer and probe validation experiment.

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference housekeeping gene must be approximately equal. This can be established by looking at how ΔCt_{Sample} varies with template dilution. If the gradient of cDNA dilution versus ΔCt_{Sample} is close to zero (<0.1) (343) it implies that the amplification efficiencies of the target and housekeeping genes are similar.

The validation experiments were performed using a 1 in 2 serial dilution for the input template (cDNA). Seven dilutions were performed ranging from 100ng to 1.56 ng. At least five dilution points were used for future analysis. The same breast biopsy was used to perform the validation experiment for all genes of interest. Two housekeeping genes (18S rRNA and HPRT) were tested for this study (Results 3.1).

2.2 In Vitro Studies

Different types of immortalized tumourogenic human mammary cell lines (MCF-7, MDA-MB-453 and MDA-MB-231) were used in this study as described below. These cell lines were obtained form American Tissue Culture Collection ATCC / European Collection of Cell Cultures ECACC). These cell lines represented both ER positive and negative breast cancers, and expressed varying degrees of the tyrosine kinase receptors; epidermal growth factor receptor HER 2. (Table 2.5) shows the ER and RTK status, growth properties and morphology of the cell lines used.

2.2.1 Culturing the breast cancer cell lines.

MCF-7, MDA-MB-453 and MDA-MB-231 cells were grown in Dulbecco's Modified Eagle Medium **(**DMEM) (Lonza) with 4.5 g/L glucose. Growth media were supplemented with heat-inactivated foetal calf serum (10%) (Invitrogen), 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin (50 units/ml, 50 μg/ml) (Invitrogen, UK). Cells were maintained in 5% CO2 at 37°C, with their medium changed regularly; they were grown in T-75 flasks (Fisher Scientific, UK**).**

Table 2.5: ER & HER status of breast cancer cell lines. Three breast cancer lines were used in the course of this research: - MCF-7, MDA-MB-231 and MDA-MB-453.

Cell Line	Growth properties	Morphology	ER Status	HER2 Status
MCF-7	Adherent	Epithelial	+	+
MDA-MB-231	Adherent	Epithelial	-	-
MDA-MB-453	Adherent	Epithelial	-	+

2.2.2 Trypsinisation of Cells.

Cells were routinely passaged to prevent cells becoming over-confluent and forming clumps. In order to split the growing cells either for passaging and/or seeding for the

subsequent experiment, old medium was removed from the flasks and the cells washed with warmed Hank's Balanced Salt Solution (HBSS) without Ca^{+2}, Mg^{+2} and phenol red (Lonza). Then Trypsin EDTA (Lonza) was added to the flask (3mls Trypsin per T-75 flask). Cells were incubated with trypsin for 3-5 minutes in 5% CO_2, 37°C, in order to detach the cells from the flask. Once cells were no longer adherent, twice the volume of growth medium was added to inactivate trypsin. Cells were gently pipetted up and down to dislodge any cell clumps that may have formed to produce a single cell suspension to allow for an accurate cell count using a haemocytometer. Having counted the number of cells present within the suspension cells were seeded into new flasks containing 10mls of new DMEM at the required density, depending on the cell line being used and the experiment.

2.2.3 Establishing frozen cell stocks.

Having trypsinised the cells, aliquots of the cells could be stored for future use. The cell suspension was transferred from the flask to a 25 ml centrifuge tube and centrifuged at 1200 rpm for ten minutes to form a cell pellet. The medium was removed and the pellet resuspended gently in 1 ml of appropriate medium containing 10% of Dimethyl sulfoxide (DMSO) (Sigma, UK). The cell suspension was immediately transferred to a 1.8 ml polypropylene cryo-vial and placed at -80°C overnight before being transferred to liquid nitrogen (-180°C) for long-term storage.

If these cell aliquots were required at a later date, they were removed from liquid nitrogen and rapidly thawed in a 37°C water bath. Cell clumps were dispersed by pipetting and were transferred to a flask containing 10mls of pre-warmed growing medium.

2.2.4 Immunofluorescence (IF) technique.

Immunofluorescence (IF) is a technique that permits the detection of a specific protein or antigen in cells or tissue sections by fluorescent visualisation. As with immunohistochemistry, there are two types of immunofluorescence staining methods, direct and indirect immunofluorescence.

The first requires using a primary antibody labelled with fluorescent dye, whereas the indirect approach involves the use of a fluorescent secondary antibody specific for immunoglobulin of the species in which the primary antibody was raised. The protein of interest is then visualised using a fluorescent microscope. This indirect method was used to investigate the expression of ER in breast cancer cell lines.

Approximately 3000 MCF-7 or MDA-MB-231 or MDA-MB-453 cells were seeded onto 6 well culture slides (VWR) and grown for three days. On day 4, serum and phenol-red free medium was removed and cells washed twice with ice-cold PBS, before being fixed to the culture slides by incubation in ice-cold methanol (500 μl of methanol per well) for three minutes. Excess methanol was removed by washing the cells three times in dH_20 for five minutes. To reduce non-specific binding of the antibody, cells were incubated in 500 μl of 1.5% normal horse serum ((Vector Laboratories, CA, USA)) in antibody diluent (DAKO) for twenty minutes. As of this point all steps were performed on an orbital shaker and at room temperature.

Cells were incubated with ERα (0.2 mg/ml, DAKO) antibody for 1 hour (100 μl of antibody per well) before being washed three times with TBS for ten minutes. Following this, cells were incubated with 100μl biotinylated mouse secondary antibody (3 μg/ml in antibody diluent) (Vector Laboratories) for thirty minutes. Cells were again washed three times with TBS for ten minutes, before being treated with 100 μl of Fluorescein Avidin D (diluted 1:100 in TBS) (Vector Laboratories) for thirty minutes. Fluorescein Avidin D has an excitation at 495 nm and an emission at about 515 nm; therefore treated cells fluoresced green. Once the cells were treated with the fluorescent protein, slides were covered in foil and all future steps performed in semi-darkness, to prevent the fluorescence from fading. Finally cells were washed with TBS three times for ten minutes. At this stage culture slides were either mounted onto coverslips, or the procedure continued to enable detection of a second protein.

2.2.5 Protein extraction and Western blotting.

2.2.5.1 Protein Extraction

Protein was extracted from growing cells after the trypsinisation step. From this point onwards all treated tubes were kept on ice. The cell pellet was dislodged by gently pipetted up and down in the ice-cold PBS. Then the cells were centrifuged at 1200 rpm for 5 minutes to form a cell pellet again. This step was repeated three times to remove any trace of growth medium proteins. It was important to remove as much of the PBS after washing, using a pipette, since it would dilute the lysis buffer. The next step involved lysing the cells to enable collection of the protein. This was done using cell lysis buffer (Cell Signaling Technology) and a non-denaturing method. The buffer used was comprised of the following: 20 mM Tris-HCl (pH 7.5), 150mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium

orthovanadate (Na_3VO_4), and 1μg/ml leupeptin. Directly before use, this buffer was diluted ten times in dH_20 and 1 mM phenylmethlysulfonyl fluoride (PMSF), a serine protease inhibitor, added. This prepared buffer was stored on ice at all times. 500 μl of buffer was added to each 25 ml centrifuge tube and cells were incubated in the buffer on ice for five minutes. The cell lysate was transferred to an appropriately labelled 1.5 ml eppendorf tube and stored on ice or at -20°C.

2.2.5.2 Determination of protein concentration in prepared samples.

Before performing immunoblotting, it was necessary to determine the concentration of protein present in the samples to be tested. Knowing this enabled experimental consistency, as the same volume of protein was used from all the samples. The method used was Bio-Rad's protein assay, which was based on the Bradford dye-binding procedure (Bradford 1976), and involves a colorimetric assay for measuring total protein concentration. For detailed explanation see appendix 2.

2.2.5.3 Western blotting technique.

Western blotting (immunoblotting) is a technique that enables the detection and quantification of the levels of a specific protein in tissue or cell samples. The basic principle requires preparing protein samples from tissues or cells and then separating the denatured proteins by gel electrophoresis using a polyacrylamide gel and transferring them to a PVDF (polyvinylidene difluoride) membrane. The membrane is then probed with a primary antibody specific to the protein of interest, followed by a secondary antibody that recognises the antibody-antigen complex. Proteins are detected using a chemiluminescent method.

Proteins from MCF-7, MDA-MB 231and MDA-MB-453 cells were resolved by 10% SDS-PAGE at 40mA for 1 hour and transferred to PVDF membrane overnight at 10V. The membrane was treated with 5% Non-Fat Dry Milk/ in TTBS (Tris Buffered Saline-Tween) for 1 hour and incubated with primary antibody (ERα, 0.2 μg/ml, DAKO, Glostrup, Denmark) or (Tubulin 1:1000 Cell Signalling Technology, Rabbit) overnight at 4^oC. Membranes were incubated in appropriate secondary antibody for 1 hour, anti-mouse IgG (CST, 1:10000) for ERα or and anti-Rabbit IgG (CST, 1:5000) for tubulin and visualised using ECL Plus chemiluminescent Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK), and detected on autoradiography film.

To avoid re-running of samples, primary antibody was removed from probed membranes using Restore Western Blot Stripping Buffer (Pierce). Membranes were incubated in 20 mls of stripping buffer at 37°C for 15 minutes, before being washed in TTBS three times for 10 minutes. Consequently, membranes were blocked in 5% Non-Fat Dry Milk/TTBS and then probed with the next primary and secondary antibody as described above.

2.2.6 Cell Proliferation Assay WST-1.

Cellular proliferation, after the different treatments used in this thesis, was determined using a non-radioactive, spectrophotometric quantification of cell proliferation and viability, by the WST-1 assay. The WST-1 assay depends on the cleavage of the tetrazolium salt WST-1, to formazan, by mitochondrial dehydrogenases. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This augmentation in enzyme activity leads to an increase in the amount of formazan dye produced, which correlates directly to the number of metabolically active cells in the culture.

The formazan dye produced by the proliferating cells was measured at an absorbance of 450 nm by spectrophotometry (Multiskan EX, Thermo, Electron Coporation). A reference reading of an absorbance at wavelength of 600nm was subtracted from the reading for each well.

10 μL of cell proliferation reagent WST-1 (Roche) was added per well, to the cells already cultured in 100 μL per well (1 in 10 final dilution). Readings were normalized to a blank control that has no growing cells and contains all reagents. Control wells that contain untreated experimental cells were included in every experimental plate.

Cell proliferation was measured in relation to an untreated control that represents 100% proliferation at the selected time point as per the following equation

$$\text{Cell proliferation } \% = \frac{\text{Absorbance reading for tested cells}}{\text{Absorbance reading for untreated control}} \times 100$$

The optimal incubation period was determined after measuring the absorption repeatedly, at different time points of incubation following the addition of WST-1 (0.5, 1, 2 and 4 hrs). 4 hrs proved to be the optimal incubation period that was associated with higher absorbance reading (Figure 2.5).

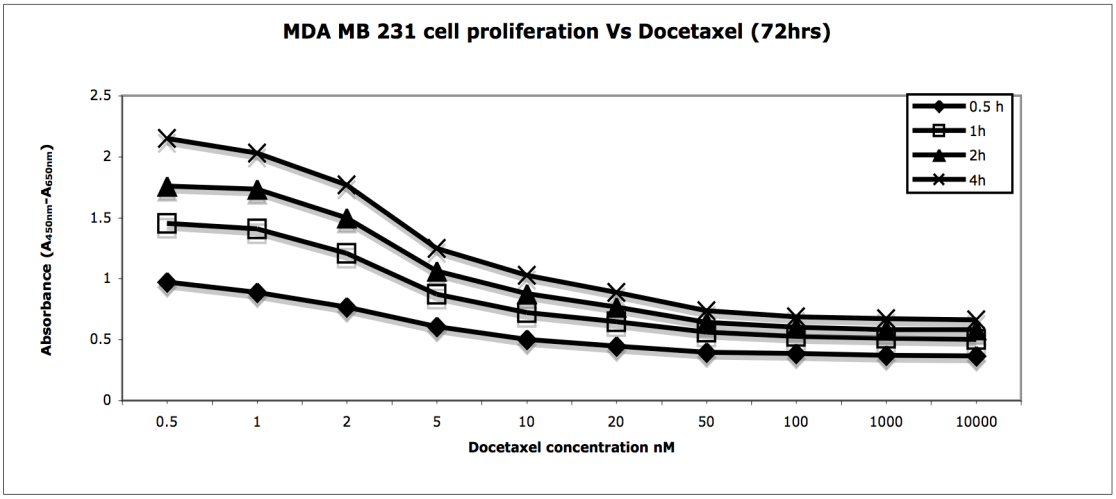

Figure 2.5: Optimal incubation period for WST assay.

The MDA-MB-231 cells were treated with different concentration of Docetaxel for 72 hours. Absorbance (A_{450nm}-A_{650nm}) was measured repeatedly at different time point of incubation after adding the WST-1 (0.5, 1, 2 and 4 hrs). Higher reading was associated with 4hrs of incubation and has been considered as an optimal incubation period.

2.3 Methodology of the drug experiments.

2.3.1 Docetaxel.

Docetaxel is a taxane based anti-cancer agent and part of a relatively new anticancer drugs group. These are classified as mitotic poisons that are commonly used as cytotoxic agents in treating metastatic and locally advanced breast cancers, especially ER^{-ve} tumours. It binds to and stabilizes the β−tubulin subunit of microtubules, preventing depolymerization of the mitotic spindle thus leading to cell cycle arrest and apoptosis.

Since treatment with Docetaxel is mainly indicated in patients with advanced ER^{-ve} breast cancers, an established ER^{-ve} mammary cancer cell line (MDA-MB-231) was selected for this experiment.

Docetaxel (C43 H53 NO14, Molecular weight 807.88 g) was purchased from Sigma-Aldrich, UK. An initial solution of 5mM Docetaxel was prepared in 100% DMSO and stored at 2-8°C, from which a stock solution of 50μM was prepared in (1% DMSO/ 99% HBSS) and stored at 2-8°C. The latter was used to prepare all other concentrations of Docetaxel in the appropriate growth media.

2.3.1.1 MDA-MB-231 cell number titration.

First a cell number titration experiment was performed to determine the optimal number of MDA-MB-231 cells to be seeded per well in the 96-well plate for this experiment. MDA-MB-231 cells were seeded at four different numbers per well (2500, 5000, 7500, and 10000) for three different time periods of 3, 4, and 5 days. The experiment with the MDA-MB-231 cells involves 3 days of incubation with Docetaxel. In order to prepare for a future study of the response of Sirtuin genes to Tamoxifen in the MDA-MB-231 cells, the cells were also incubated for 4 and 5 days. The data showed that a cell number of between 2500 and 5000 was the best for a 3-day incubation period. Therefore in the following experiments, the number of MDA-MB-231 cells to be seeded was selected depending on the incubation period in the 96-well plate, (Results (5. 2. 2. 1).

2.3.1.2 Optimisation of Docetaxel concentration and treatment period.

The MDA-MB-231 cells were treated with 1nM, 10nM and 100nM Docetaxel. These three concentrations were decided upon following an experiment investigating the effects of various concentrations (0.5 nM, 1 nM, 2 nM, 5 nM 10 nM, 20 nM, 50 nM, 100 nM, 1000 nM) of Docetaxel on cellular proliferation. 1 nM, 10 nM and 100 nM were shown to be the optimal concentrations for the purpose of our studies, (Results (5. 2. 2. 2).

The MDA MB 231 cells were treated with Docetaxel for 72 hrs. This time was decided upon following an experiment investigating the effects of four different time points 24 hrs, 48 hrs, 72 hrs and 96 hrs) (Results (5. 2. 2. 3).The concentration of the DMSO was adjusted to be the same in all Docetaxel concentrations: 0.002%.

In each experiment a DMSO-only control was included which was used as a “Calibrator” for determining relative gene expression.

2.3.2 Tamoxifen.

Tamoxifen is commonly used for the treatment of both early and advanced Oestrogen Receptor positive (ER+ve) breast cancers, in pre- and post-menopausal women. Tamoxifen is a nonsteroidal agent, with potent antioestrogenic properties, which competes with oestrogen for binding sites in breast tumour and other tissue targets. TAM is believed to inhibit the growth of breast cancer mainly through competing with oestrogen for oestrogen receptor binding.

Tamoxifen citrate ($C_{26}H_{29}NO \cdot C_6H_8O_7$, Molecular weight 563.6 g) was purchased from Sigma-Aldrich. A stock solution of 10 mM Tamoxifen was prepared in 100% DMSO and stored at 2-8°C. This was used to prepare all other concentrations of Tamoxifen in the appropriate growth media.

Two different types of immortalized tumourogenic human mammary cell lines (MCF7, and MDA-MB-453) were used in this study as described below. The MCF7 cells were selected as they represent an established ER^{+ve} mammary cancer cell line. The MDA-MB-453 cells were selected as they represent an established ER^{-ve} mammary cancer cell line that resembles the MCF7 cells in HER2 status (Table 2.3).

2.3.2.1 MCF7 cell number titration.

A cell titration experiment was performed first to determine the optimal number of MCF7 cells to be seeded per well in the 96-well plate for this experiment. MCF7 cells were seeded at three different numbers per well (5000, 10000, and 15000 for three different time periods of 3, 4, and 5 days. The experiment with the MCF7 cells involve a maximum of 5 days incubation in the 96-well plate; 2 days in oestrogen free media and then a maximum of three days with the drug. The data showed that cell numbers between 5000 and 10000 were the best for 5 days incubation period in the 96-well plate Therefore in the following experiments, the number of MCF7 cells to be seeded was selected depending on the incubation period in the 96-well plate (Results (5. 3. 2. 1).

2.3.2.2 Optimisation of Tamoxifen concentration and treatment period in the MCF7 cell line.

The MCF7 cells were treated with 5 μM, 8 μM and 10 μM Tamoxifen. These three concentrations were decided upon following an experiment investigating the effects of various concentrations (2 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM and 12 μM of Tamoxifen on cellular proliferation. 5μM, 8μM and 10μM were shown to be the optimal concentrations for the purpose of our studies (Results (5. 3. 2. 2).

The MCF7 cells were treated with Tamoxifen for 72hrs. This time was decided upon following an experiment investigating the effects of four different time points 24hrs, 48hrs, 72hrs and 96hrs) (Results (5. 3. 2. 3).

2.3.2.3 MDA-MB-453 cell number titration.

A cell titration experiment was performed first to determine the optimal number of MDA-MB-453 cells to be seeded per well in the 96-well plate for this experiment. MDA-MB-453 cells were seeded at four different numbers per well (5000, 10000, 15000 and 20000 for three different time periods of 3, 4, and 5 days. The experiment with the MDA-MB-453 cells involve a maximum of 5 days incubation in the 96-well plate; 2 days in oestrogen-free media and then a maximum of three days with the drug. The data showed that cell number equal to 5000 was the best for 5 days incubation period in the 96-well plate. Therefore in the following experiments, the number of MDA-MB-453 cells to be seeded was decided depending on the incubation period in the 96-well plate Optimisation of Tamoxifen concentration and treatment period in the MDA-MB-453 cell line.

The MDA-MB-453 cells were treated with 5 μM, 8 μM and 10 μM Tamoxifen. These three concentrations were decided upon following an experiment investigating the effects of various concentrations (2 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM and 12 μM of Tamoxifen on cellular proliferation. 5 μM, 8 μM and 10 μM were shown to be the optimal concentrations for the purpose of our studies (Results (5. 4. 2. 2).

The MDA-MB-453 cells were treated with Tamoxifen for 72 hrs. This time was decided upon following an experiment investigating the effects of four different time points 24hrs, 48hrs, 72hrs and 96hrs) (Results (5. 4. 2. 2).

2.3.3 Tamoxifen treatment: experimental design.

All experiments were carried out in duplicate using the same cell preparation, media and treatments in parallel.

In all Tamoxifen experiments cell lines were treated as follows:

- Cells were incubated in phenol-free media supplemented with 5% charcoal stripped foetal calf serum for 48h before being treated with Tamoxifen for the tested period to eliminate the effect of oestrogen on cellular growth.

- Cells treated for 72 hours using the three selected concentrations of Tamoxifen, in phenol-free media supplemented with 5% charcoal stripped fetal calf serum and 1nM β-estradiol (Sigma Aldrich, uk) .

- The concentration of the DMSO was adjusted to be the same in all experiments: 0.1%.

- In each experiment a DMSO-only control was included which was used as a "Calibrator" for determining relative gene expression.

Chapter III

The relative transcriptional expression of sirtuin genes in breast cancer

3.1 Establishment of Real time PCR procedure.

3.1.1 Primer and probe validation experiment.

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference housekeeping gene must be approximately equal. This can be established by looking at how ΔCt_{Sample} varies with template dilution. The ΔC_T value was plotted against the logarithm (Log) of the template amount. If the efficiencies in generating the two amplicons are approximately equal, the slope value of the plot will be approximately zero. The recommended value for the slope of log input amount vs. ΔC_T is < 0.1 (343).

The validation experiments were performed using a 1 in 2 serial dilution for the input template (cDNA). Seven dilutions were performed ranging from 100 ng to 1.56 ng. At least five dilution points were used for future analysis. The same breast biopsy was used to perform the validation experiment for all genes of interest. Two housekeeping genes,18S rRNA (18S) and HPRT, were tested for this study.

3.1.1.1 Validation experiments of SIRT4, SIRT5, and SIRT6 primers and probes in relation to 18S rRNA.

The validation experiments were performed first for SIRT4, SIRT5 and SIRT6 primers and probes in relation to the 18S housekeeping gene (Figure 3.1). The slope values for SIRT4, SIRT5 and SIRT6 in relation to 18S were much greater than 0.1: 0.38, 0.42 and 0.73, respectively (Figure 3.1). These data indicated that the 18S was not suitable as a housekeeping gene for this experiments. Further analysis for the remaining Sirtuin genes in relation to the 18S housekeeping gene was not performed.

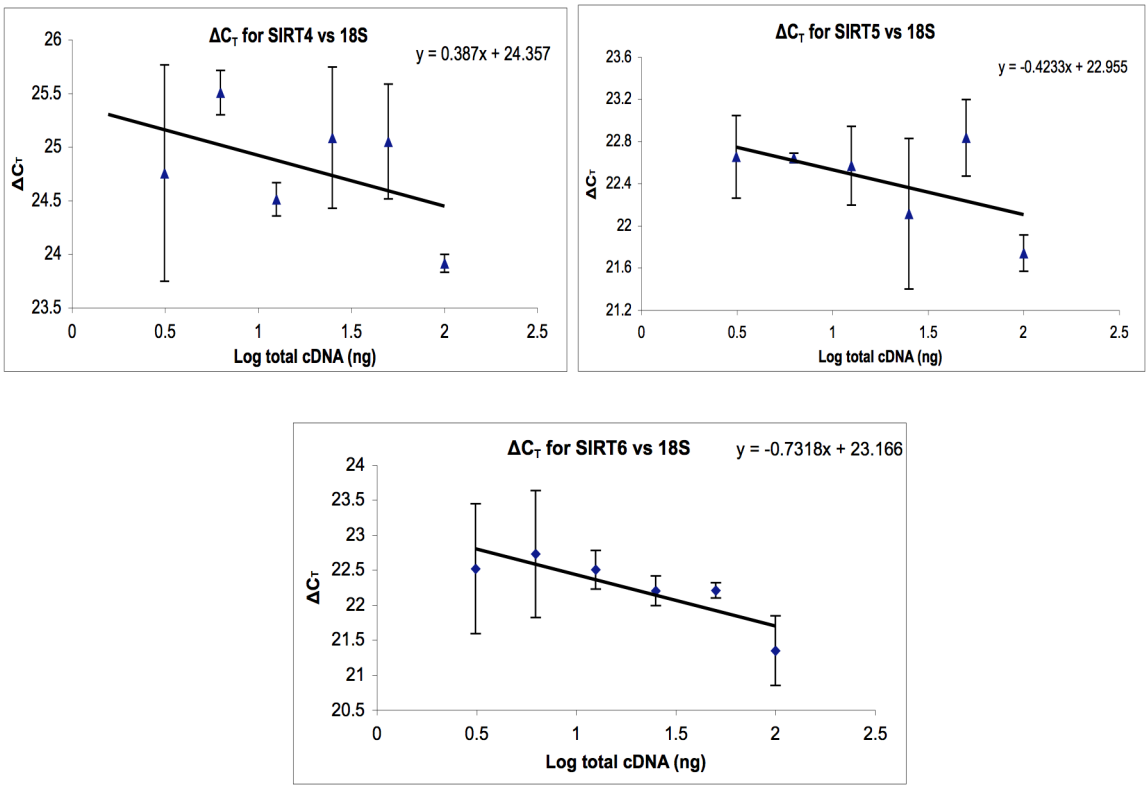

Figure 3.1: Validation experiments for SIRT4, SIRT5 and SIRT6 primers and probes in relation to the 18S housekeeping gene.

Graphs show the variation of ΔCt value for SIRT4, SIRT5 and SIRT6 in relation to a *18S* housekeeping gene when template input changes. The x axis represents the $\log_{10}$ of the input amount of cDNA, whereas the y axis represents the ΔCt value. It is important for the $2^{-\Delta Ct}$ method to be valid, to confirm that the amplification efficiencies are equal for both gene of interest and housekeeping gene in the range of template input used. This is confirmed when the slope value is less than 0.1.

3.1.1.2 Validation experiments of sirtuin primers and probes in relation to HPRT.

The ΔCt values for all sirtuin primers and probes in relation to HPRT as the internal reference gene were acceptable when the amount of input cDNA was between 100 ng and 12.5 ng (Figure 3.2). The slope values of: 0.05 for SIRT1, 0.003 for SIRT2, 0.095 for SIRT3, 0.008 for SIRT4, 0.081 for SIRT5, 0.063 for SIRT6 and 0.052 for SIRT7 were indicative of equal efficiencies in generating the two amplicons using these sets of primers and probes for the gene of interest (sirtuin) and housekeeping gene (HPRT) compared together in the tested range of input cDNA (Figure 3. 2).

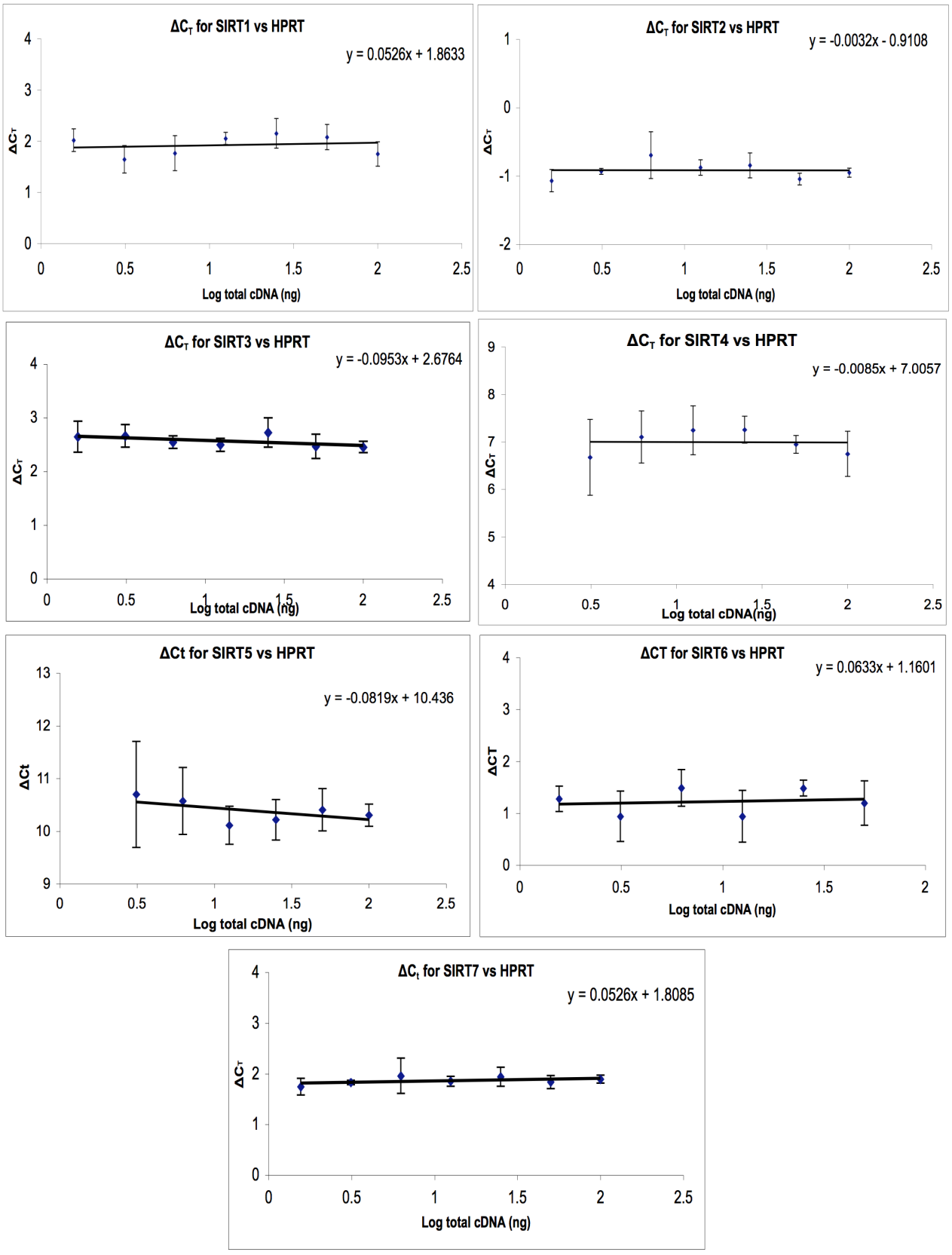

Figure 3.2: Validation experiments for sirtuin primers and probes in relation to the HPRT housekeeping gene.

Graphs show the variation of ΔCt value for sirtuins in relation to a *HPRT* housekeeping gene when template input changes. The x axis represents the $\log_{10}$ of the input amount of cDNA, whereas the y axis represents the ΔCt value. It is important for the $2^{-\Delta\Delta Ct}$ method to be valid, to confirm that the amplification efficiencies are equal for both gene of interest and housekeeping gene in the range of template input used. This is confirmed when the slope value is less than 0.1.

3.1.1.3 Validation experiments of XRCC5, p21, BCL2 and KI67 primers and probes in relation to HPRT.

The ΔCt values for XRCC5, p21, BCl2, KI67 primers and probes in relation to HPRT were acceptable when the amount of input cDNA was between 100 ng and 12.5 ng. The slope values of: 0.081 for XRCC5, 0.007 for p21, 0.064 for BCL2, and 0.009 for KI67 were indicative of equal efficiencies in generating the two amplicons using these sets of primers/probes for XRCC5, p21, BCl2, KI67 and the housekeeping gene (HPRT) compared together in the tested range of input cDNA (Figure 3.3).

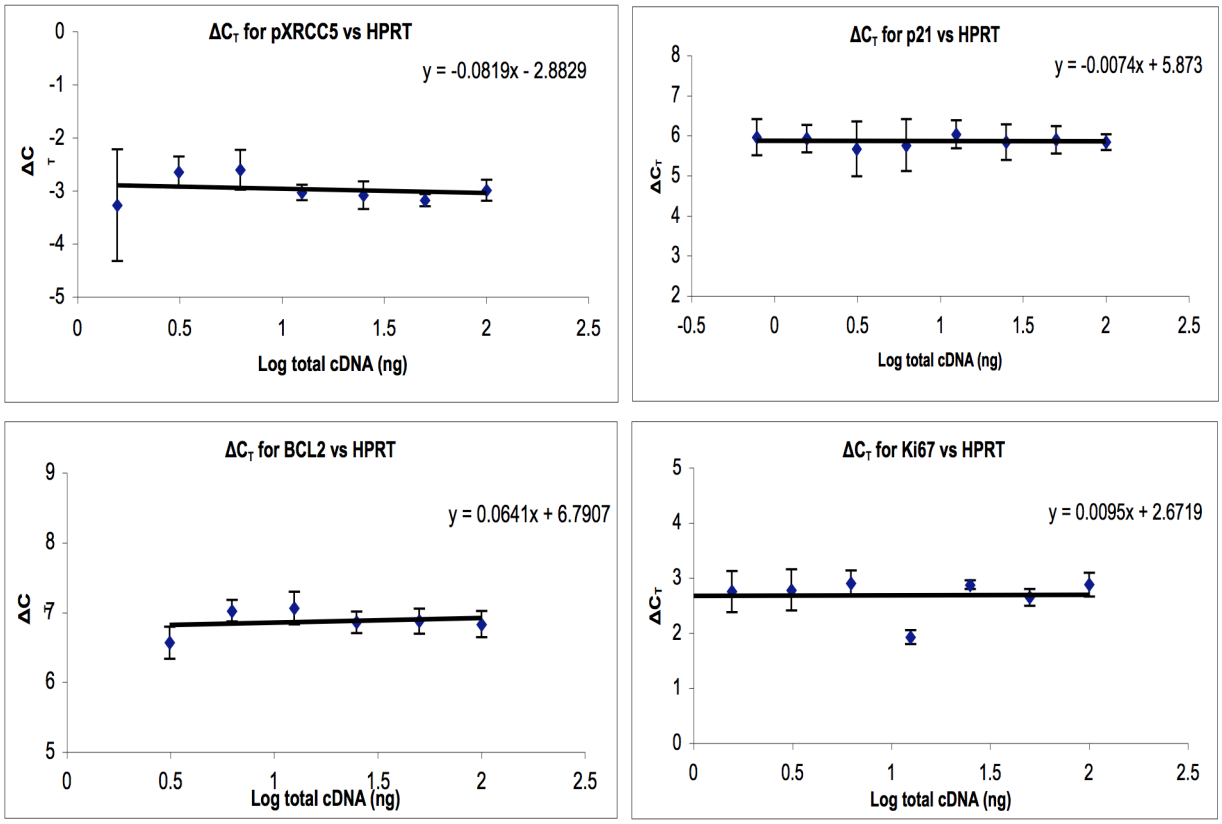

Figure 3.3: Validation experiments for XRCC5, p21, BCl2, and KI67 primers and probes in relation to the HPRT housekeeping gene.

Graphs show the variation of ΔCt value for sirtuins in relation to a *HPRT* housekeeping gene when template input changes. The x axis represents the $\log_{10}$ of the input amount of cDNA, whereas the y axis represents the ΔCt value. It is important for the $2^{-\Delta\Delta Ct}$ method to be valid, to confirm that the amplification efficiencies are equal for both gene of interest and housekeeping gene in the range of template input used. This is confirmed when the slope value is less than 0.1.

3.1.2 Conclusion.

Two housekeeping genes (18S rRNA and HPRT) were tested for this study. The validation experiment results indicate that HPRT is a better housekeeping gene for determining the relative transcriptional expression of all sirtuin, BCL2, Ki67, XRCC5 and p21 genes under

these experimental conditions. Therefore, HPRT was selected as a housekeeping gene and a template input between 25 ng-50 ng was used for the subsequent Taqman experiments.

3.2 Introduction.

In this experiment the relative transcriptional expression of sirtuins 1-7 was investigated, by Real time PCR technique in a sample of archival breast biopsies, obtained from 177 women. These comprised normal breast biopsies (n = 25) from patients undergoing breast reduction surgery (reduction mammoplasty), non-malignant (n = 78) and malignant (n = 73) breast biopsies, obtained after routine histopathological analysis from patients who had undergone breast surgery for potentially curable, invasive ductal adenocarcinomas, between 1987 and 2000.

This study aimed to investigate whether the relative transcriptional expression for sirtuins 1-7 was altered in breast tumour biopsies when compared to both the normal and non-malignant biopsies. Furthermore, it sought to determine if there were any associations between sirtuin expression levels, clinico-pathalogical features of the disease, including histopathological parameters (tumour size, tumour grade, nodal status and oestrogen receptor status and lymph-vascular invasion) and patient's outcome.

3.3 Characteristics of the studied patient cohorts.

3.3.1 Age distribution in the patient cohorts.

Age was normally distributed in all patient groups (normal, non-malignant and malignant; Figure 3.4). The median age was 41.6 years (20 years – 63 years) for the normal group, 61years (32 years - 84 years) for the non-malignant group and 61 years (33 years - 94 years) for the malignant group (Figure 3. 5). Pairwise comparisons of the three groups using the Mann-Whitney test showed that there was no significant difference in age between the non-malignant and malignant groups ($p = 0.223$). However, age in the normal group was significantly different from the age in both non-malignant and malignant groups ($p < 0.001$; Figure 3.5).

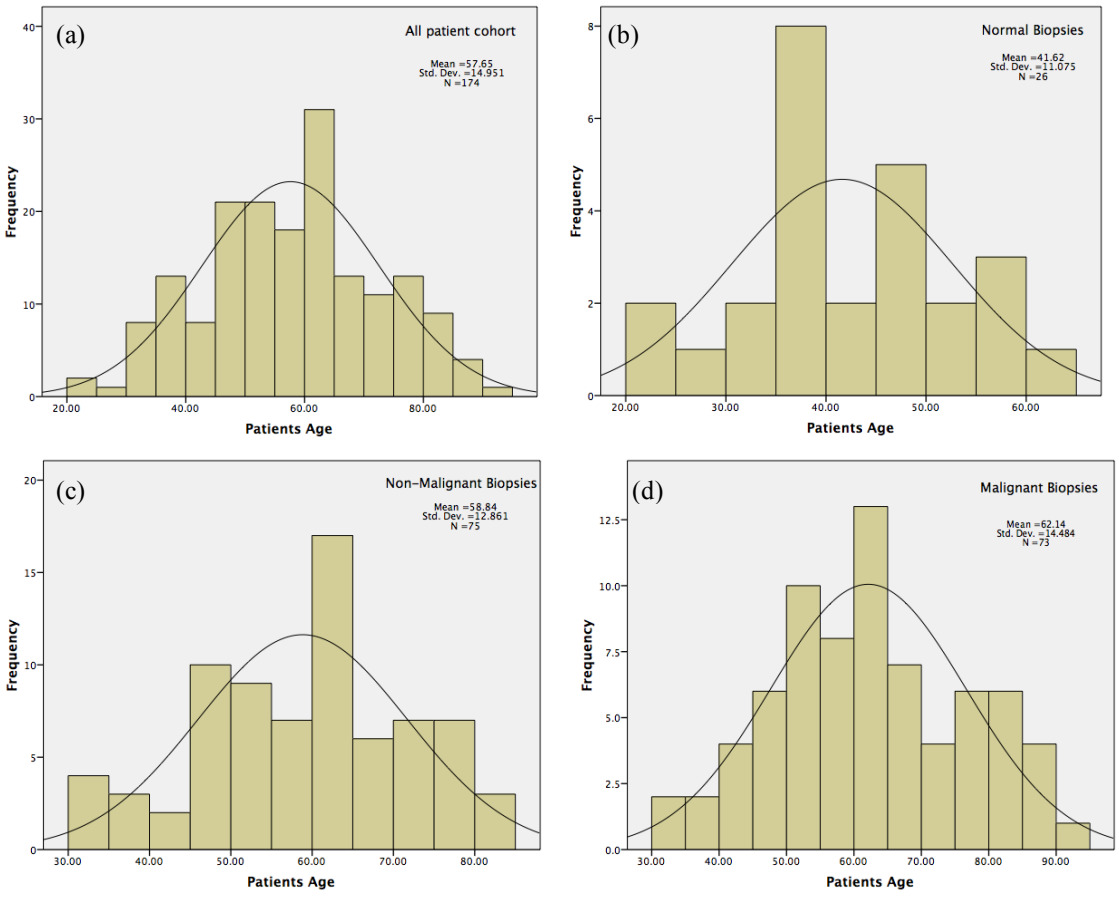

Figure 3.4: Distribution of patient's age in the three different groups.

(a) patient's age in the entire patient cohort, (b) patient's age for the normal breast biopsy group, (c) patient's age for the non-malignant breast biopsy group, (d) patient's age in for the malignant breast biopsy group.

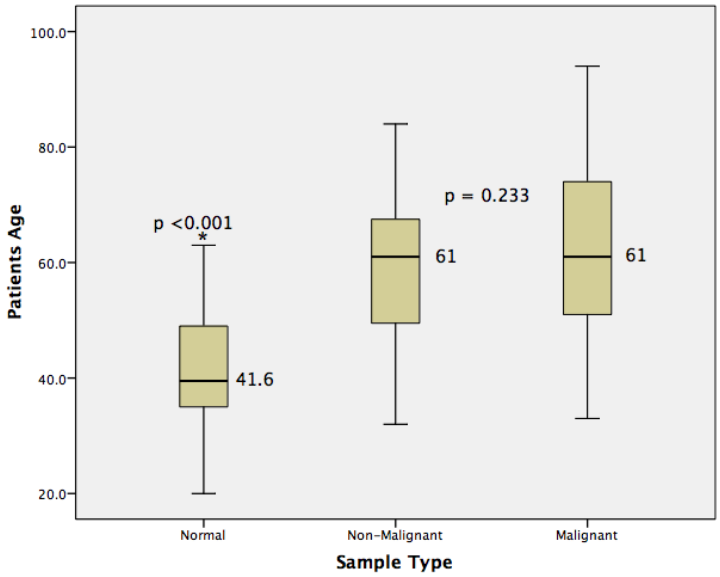

Figure 3.5: Box plot showing patient's age in normal, non-malignant and malignant breast biopsy groups.

The median value for each patient group is shown in the clear box to the right of each box plot.

3.3.2 Survival according to the known prognostic markers.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to the known prognostic markers; namely tumour grade (G), tumour size (T), nodal status (N^{-ve} and N^{+ve}), oestrogen receptor status (ER^{-ve} and ER^{+ve}), Lymphovascular invasion status (LV^{-ve} and LV^{+ve}) and Nottingham Prognostic Index (NPI). Patient numbers included in each patient group are displayed in (Table 3.1). Furthermore, Cox-Regression analysis was calculated for all known prognostic markers and is shown in (Table 3.1).

Table 3.1: Number of breast cancer patients and cancer specific deaths of all patient groups included in the Kaplan-Meier survival analysis according to the known prognostic markers.

p values for both Kaplan-Meier and Cox-Regression analyses are also shown.

	Prognostic factors in breast cancer															
	Tumour Grade (G)			Tumour size (T)				Nodal		ER status		LV status		NPI		
	G1	G2	G3	T1	T2	T3	T4	N^{-ve}	N^{+ve}	ER^{-ve}	ER^{+ve}	LV^{-ve}	LV^{+ve}	<3.4	3.4-5.4	>5.4
Patients Number	4	30	31	10	45	13	2	20	43	21	32	19	28	3	37	19
Number of Cancer specific deaths	1	10	9	3	10	6	1	2	17	7	10	5	7	0	10	9
Kaplan-Meier p	p = 0.893			p = 0.072 p = 0.013				p = 0.003		p = 0.833		p = 0.99		P = 0.015		
Cox Regression p	p = 0.909			p = 0.111				p = 0.01		p = 0.834		p = 0.996		p = 0.003		

3.3.2.1 Survival analysis according to tumour grade.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to tumour grade (G). Patient numbers included in each patient group are displayed in (Table 3. 1). The Kaplan-Meier test showed that there was no significant difference in the survival period when patients were categorised according to tumour grade. The mean survival period was different in patients with G1 tumours (n = 4, 12.79 years, 95% C.I. 9.73 -15.85), when compared with both patients with G2 tumours (n = 30, 10.83 years, 95% C.I. 8.86 -12.78) and with G3 tumours (n = 31, 10.94 years, 95% C.I. 8.71 -13.17). However, this difference was not significant (Kaplan-Meier, p=0.893, Figure 3. 6).

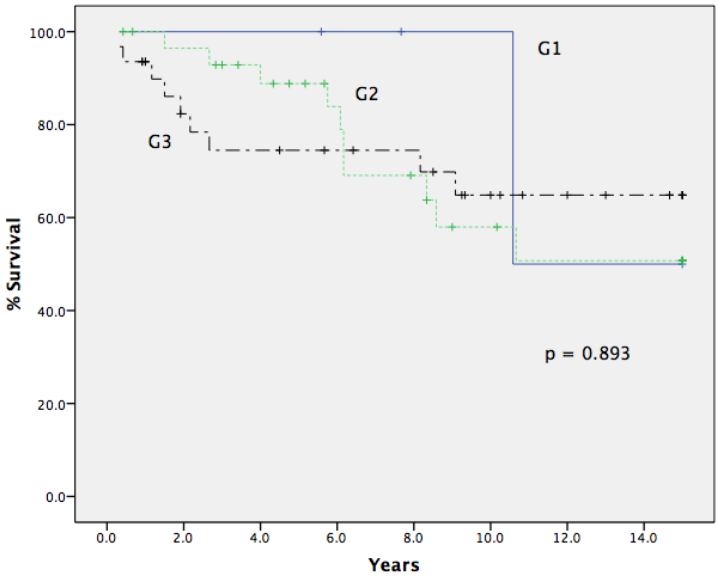

Figure 3.6: Kaplan-Meier survival plot for breast cancer patients according to tumour grade.

The Kaplan-Meier survival analysis showed that there was no significant difference in the mean survival period in patients with G1 tumours (n = 4, 12.79 years, 95% C.I. 9.73 -15.85), when compared with both patients with G2 tumours (n = 30, 10.83 years, 95% C.I. 8.86 -12.78) and with G3 tumours (n = 31, 10.94 years, 95% C.I. 8.71 -13.17; p=0.893).

3.3.2.2 Survival analysis according to tumour size.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to tumour size (T). Patient numbers included in each patient group are displayed in Table 3.2. The Kaplan-Meier test showed that there was no significant difference, but a strong trend (p = 0.072,) in the survival period when patients were categorised according to tumour size (Figure 3. 7). The mean survival period was shorter in patients with larger tumour size T3 (n = 13, 8.28 years, 95% C.I. 4.75 -11.82), and T4 (n = 2, 8.16 years),

when compared with patients with smaller tumour size T1 (n = 10, 10.27 years, 95% C.I. 6.49 -14.04) and with T2 tumours (n = 45, 12.19 years, 95% C.I. 10.69 -13.7).

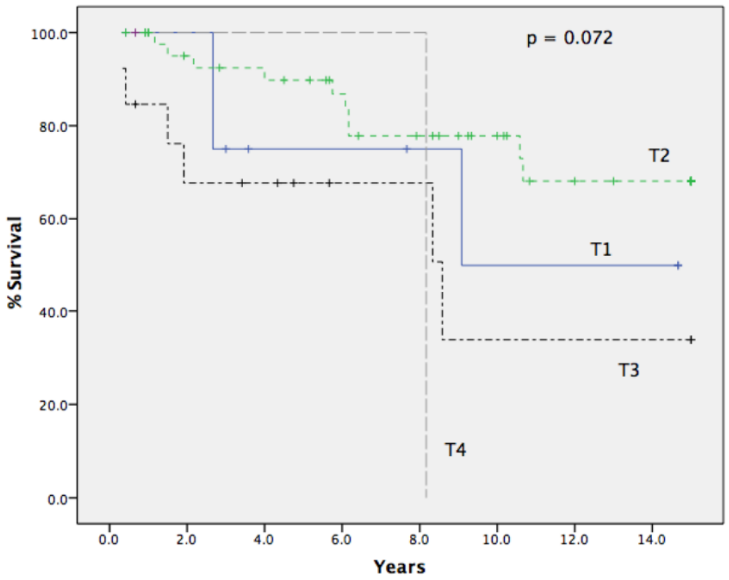

Figure 3.7: Kaplan-Meier Survival plot for breast cancer patients according to tumour size.

The Kaplan-Meier test showed that there there was no significant difference (p = 0.072) in the survival period when patients were categorised according to tumour size. The mean survival period was shorter in patients with larger tumour size T3 (n = 13, 8.28 years, 95% C.I. 4.75 -11.82), and T4 (n = 2, 8.16 years), when compared with patients with smaller tumour size T1 (n = 9, 10.27 years, 95% C.I. 6.49 -14.04) and with T2 tumours (n = 45, 12.19 years, 95% C.I. 10.69 -13.7).

As can be seen from Table 3.1 there are very few patients in both the T1 and T4 groups, and this could explain the previous result (trend toward significance only). Therefore, patients were divided into two groups; those with small tumours (T1 and T2) and those with large tumours (T3 and T4). The Kaplan-Meier test showed that the mean survival period was significantly shorter in patients with large tumours (n = 15, 8.13 years, 95% C.I. 10.48 -13.34) when compared with those with small tumours (n = 54, 11.91 years, 95% C.I. 10.49 -11.36, Kaplan-Meier, p = 0.013, Figure 3. 8). Furthermore, Cox Regression analysis of the Hazard Ratio (HR) showed that patients with large tumours was 1.11 times (95% CI 1.20 – 7.7, p =0.013) more likely to die as a consequence of the breast cancer disease when compared to patients with small tumours.

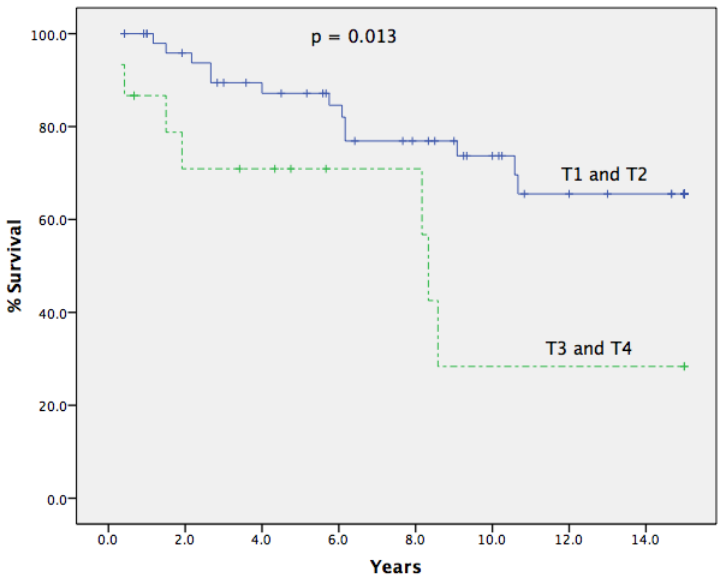

Figure 3.8: Kaplan-Meier Survival plot for breast cancer patients according to tumour size.

Patients were divided into two groups; a) patients with small tumours, T1 and T2 combined; b) patients with large tumours, T3 and T4 combined. The Kaplan-Meier test showed that the mean survival period was significantly shorter in patients with large tumours (n = 15, 8.13 years, 95% C.I. 10.48 -13.34) when compared with those with small tumours (n = 54, 11.91 years, 95% C.I. 10.49 -11.36, Kaplan-Meier, p=0.013).

3.3.2.3 Survival analysis according to nodal status.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to nodal status (N^{-ve} and N^{+ve}). Patient numbers included in each patient group are displayed in (Table 3.1). The Kaplan-Meier test showed that there was a significant difference in the survival period when patients were categorised according to nodal status (Figure 3.9). The mean survival period was shorter in patients with node positive tumours (n = 43, 9.32 years, 95% C.I. 7.38 -11.26) when compared with those with node negative tumours (n = 20, 14.18 years, 95% C.I. 13.13 -15.24, Kaplan-Meier p = 0.003). Furthermore, Cox Regression (Hazard Ratio) analysis showed that the relative risk of death was almost two times more (1.94) in patients with node positive tumours (95% CI 1.6 – 30, p=0.01) when compared with patients with node negative tumours.

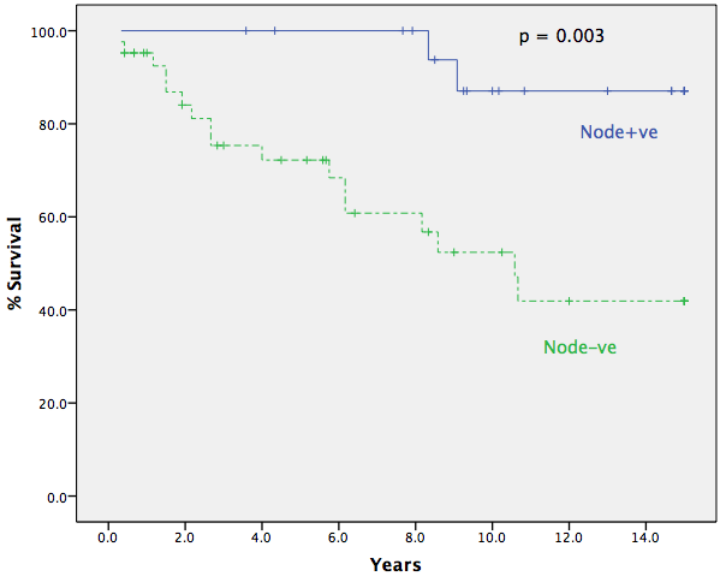

Figure 3.9: Kaplan-Meier Survival plot for breast cancer patients according to nodal status.

The Kaplan-Meier test showed that there was significant difference in the survival period when patients were categorised according to nodal status.The mean survival period was shorter in patients with node positive tumours (n = 43, 9.32 years, 95% C.I. 7.38 -11.26) when compared with those with node negative tumours (n = 20, 14.18 years, 95% C.I. 13.13 -15.24, Kaplan-Meier p = 0.003).

3.3.2.4 Survival analysis according to oestrogen receptor status.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to oestrogen receptor status (ER^{-ve} and ER^{+ve}). The oestrogen receptor status was determined according to immunohistochemistry staining, performed as part of histopathological examination of the surgically removed tumours. Patient numbers included in each patient group are displayed in (Table 3.1). The Kaplan-Meier test showed that there was no significant difference in the survival period in patients with ER^{-ve} tumours (n = 21, 10.74 years, 95% C.I. 8.17 – 13.31) when compared with those with ER^{+ve} tumours (n = 32, 10.51 years, 95% C.I. 8.39 -112.64, p = 0.833, Figure 3.10).

3.3.2.5 Survival analysis according to lymphovascular invasion status.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to lymphovascular invasion status (LV^{-ve} and LV^{+ve}). Patient numbers included in each patient group are displayed in (Table 3.1). The Kaplan-Meier test showed that there was no significant difference in the mean survival period in patients with LV^{-ve} tumours (n

= 19, 11.66 years, 95% C.I. 9.24 – 14.09) when compared with those with LV^{+ve} tumours (n = 28, 11.52 years, 95% C.I. 9.32 -13.73, p = 0.99, Figure 3.11).

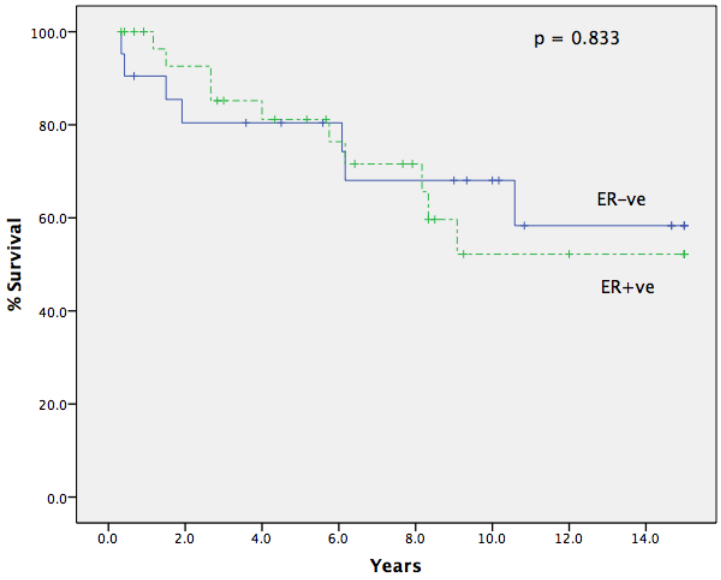

Figure 3.10: Kaplan-Meier survival plot for breast cancer patients according to oestrogen receptor status.

The Kaplan-Meier test showed that there was no significant difference in the survival period in patients with ER^{-ve} tumours (n = 21, 10.74 years, 95% C.I. 8.17 – 13.31) when compared with those with ER^{+ve} tumours (n = 32, 10.51 years, 95% C.I. 8.39 -112.64, Kaplan-Meier p = 0.833).

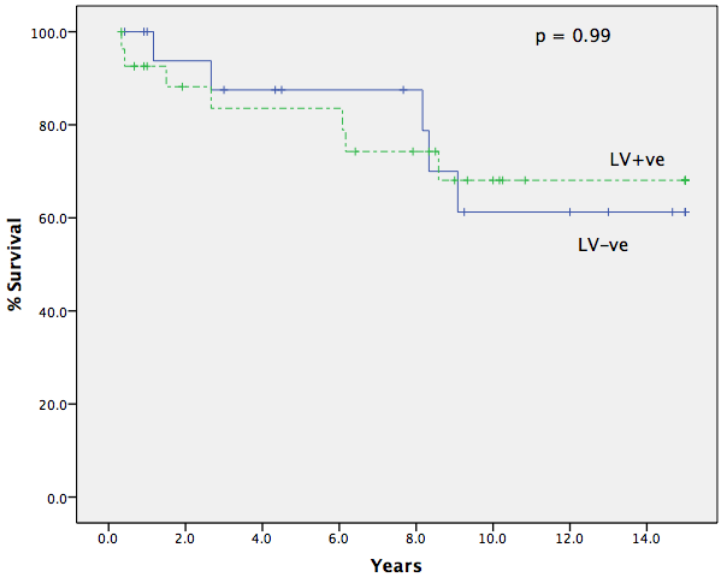

Figure 3.11: Kaplan-Meier survival plot for breast cancer patients according to lymphovascular invasion status.

The Kaplan-Meier test showed that there was no significant difference in the mean survival period in patients with LV^{-ve} tumours (n = 19, 11.66 years, 95% C.I. 9.24 – 14.09) when compared with those with LV^{+ve} tumours (n = 28, 11.52 years, 95% C.I. 9.32 -13.73, Kaplan-Meier p = 0.99).

3.3.2.6 Survival analysis according to the NPI.

The Kaplan-Meier test was used to analyse the survival data for the entire patient group according to the NPI. Patient numbers included in each patient group are displayed in (Table 3.1). The Kaplan-Meier test showed that there was a significant difference in the survival period when patients were categorised according to the NPI (p = 0.015; Figure 3.12). The mean survival period was significantly shorter in patients with high NPI (NPI >5.4) (n = 19, 7.9 years, 95% C.I. 4.48 -10.93) when compared with those with moderate NPI (NPI 3.4 - 5.4) (n = 37, 11.97 years, 95% C.I. 10.37-13.56, p = 0.015). The mean survival period could not be calculated for patients with low NPI (NPI < 3.4) because of the small number of patients and lack of complete (15 years) follow up data. Cox Regression (Hazard ratio) analysis showed that the relative risks of death in patients with high NPI was 1.25 times more (95% CI 1.47 – 8.24, p=0.004) when compared with patients with moderate NPI.

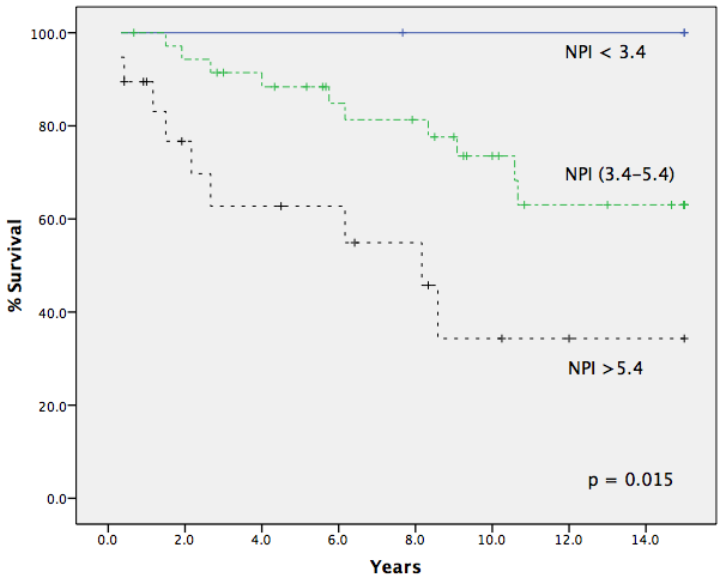

Figure 3.12: Kaplan-Meier Survival plot for breast cancer patients according to the NPI.

The Kaplan-Meier test showed that the mean survival period was significantly shorter in patients with high NPI (NPI >5.4) (n = 19, 7.9 years, 95% C.I. 4.48 -10.93) when compared with those with moderate NPI (NPI 3.4 - 5.4) (n = 37, 11.97 years, 95% C.I. 10.37-13.56, Kaplan-Meier p = 0.015).

3.3.3 Recurrence according to the known prognostic markers.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to the known prognostic markers; namely tumour grade (G), tumour size (T), nodal status (N^{-ve} and N^{+ve}), oestrogen receptor status (ER^{-ve} and ER^{+ve}), Lymphovascular invasion status (LV^{-ve} and LV^{+ve}) and Nottingham Prognostic Index (NPI). Patient numbers included in each patient group are displayed in (Table 3.2). Furthermore, Cox-Regression analysis was calculated for all known prognostic markers and is shown in (Table 3.2)

Table 3.2 : Number of breast cancer patients and tumour recurrences of all patient groups included in the Kaplan-Meier recurrence analysis according to the known prognostic markers.

p values for both Kaplan-Meier and Cox-Regression analyses are also shown.

	Prognostic factors in breast cancer															
	Tumour Grade (G)			Tumour size (T)				Nodal		ER status		LV status		NPI		
	G1	G2	G3	T1	T2	T3	T4	N^{-ve}	N^{+ve}	ER^{-ve}	ER^{+ve}	LV^{ve}	LV^{+ve}	<3.4	3.4-5.4	>5.4
Patients Number	4	30	31	10	44	13	2	20	43	20	32	19	27	3	37	19
Number of Cancer recurrences	2	10	6	3	12	3	1	2	16	6	11	4	5	0	13	4
Kaplan-Meier p	p = 0.398			p = 0.708 p = 0.637				p =0.006		p = 0.637		p = 0.771		p = 0.604		
Cox Regression p	p = 0.180			P=0.853 P=0.64				P=0.017		P=0.639		P=0.772		p=0.441		

3.3.3.1 Recurrence analysis according to tumour grade.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to tumour grade (G). Patient numbers included in each patient group are displayed in (Table 3.2). The Kaplan-Meier test showed that there was no significant difference in time to recurrence (p = 0.398) in patients with G1 tumours (n = 4, 8.87 years, 95% C.I. 2.67 -15.07), when compared with both patients with G2 tumours (n = 30, 10.57 years, 95% C.I. 9.94 -13.04) and with G3 tumours (n = 31, 12.04 years, 95% C.I. 9.94 - 14.13, Figure 3.13).

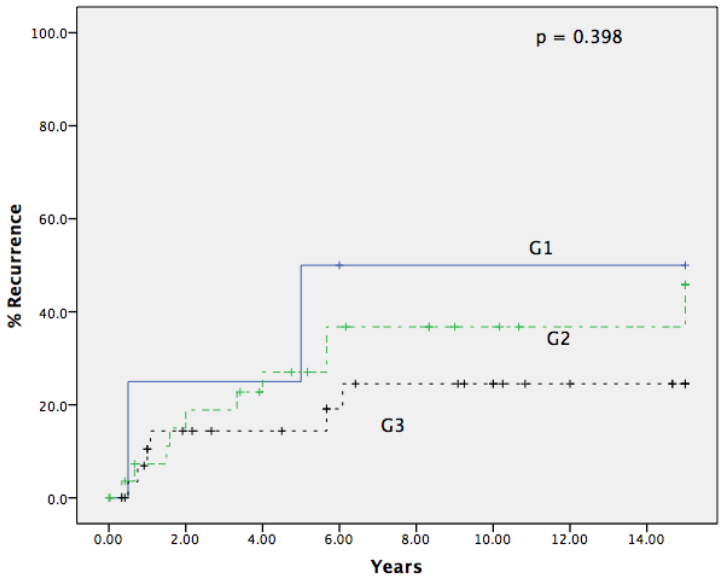

Figure 3.13: Kaplan-Meier recurrence plot for breast cancer patients according to tumour grade.

The Kaplan-Meier test showed that time to recurrence was non-significantly different (p = 0.398) in patients with G1 tumours (n = 4, 8.87 years, 95% C.I. 2.67 -15.07), when compared with both patients with G2 tumours (n = 30, 10.57 years, 95% C.I. 9.94 -13.04) and with G3 tumours (n = 31, 12.04 years, 95% C.I. 9.94 -14.13).

3.3.3.2 Recurrence analysis according to tumour size.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to tumour size (T). Patient numbers included in each patient group are displayed in (Table 3.2). The Kaplan-Meier test showed that there was no significant difference in the time to recurrence between patients with small tumour size T1 (n = 10, 9.72 years, 95% C.I. 5.28 -14.16) and T2 (n = 44, 11.53 years, 95% C.I. 9.69 -13.36) when compared with patients with larger tumour size T3 (n = 13, 10.45 years, 95% C.I. 6.29 -14.6), and T4 (n = 2, 5.66 years Kaplan-Meier p = 0.708, Figure 3.14).

A similar non-significant result was obtained after dividing the patients into two groups; patients with small tumours (T1 and T2) and patients with large tumours (T3 and T4) (Table 3.2). The Kaplan-Meier test showed that the time to recurrence was non-significantly shorter in patients with large tumours (n = 15, 9.71 years, 95% C.I. 5.82 -13.6) when compared with those with small tumours (n = 54, 11.25 years, 95% C.I. 9.53 -12.9, Kaplan-Meier, p=0.637, Figure 3.15).

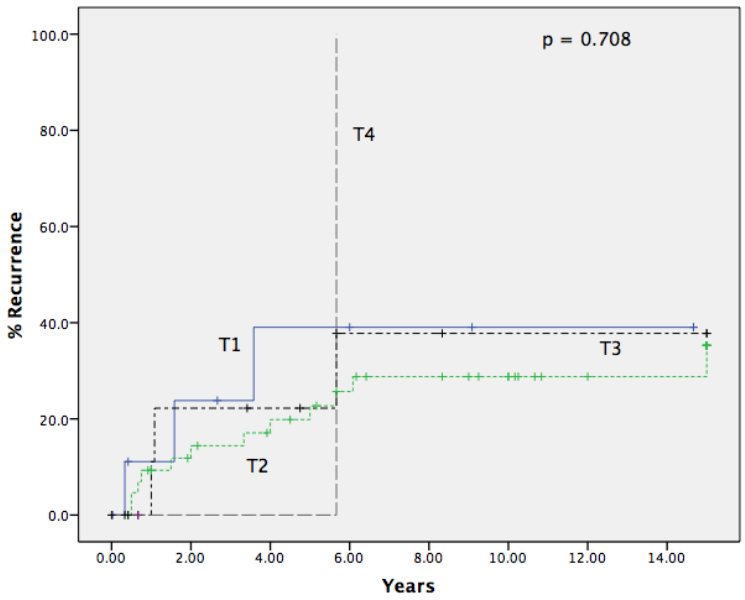

Figure 3.14: Kaplan-Meier recurrence plot for breast cancer patients according to tumour size.

Recurrence plot analysis showed that there was no significant difference in the time to recurrence between patients with small tumour size T1 (n = 9, 9.72 years, 95% C.I. 5.28 - 14.16) and T2 (n = 45, 11.53 years, 95% C.I. 9.69 -13.36) when compared with patients with larger tumour size T3 (n = 13, 10.45 years, 95% C.I. 6.29 -14.6), and T4 (n = 2, 5.66 years Kaplan-Meier p = 0.708).

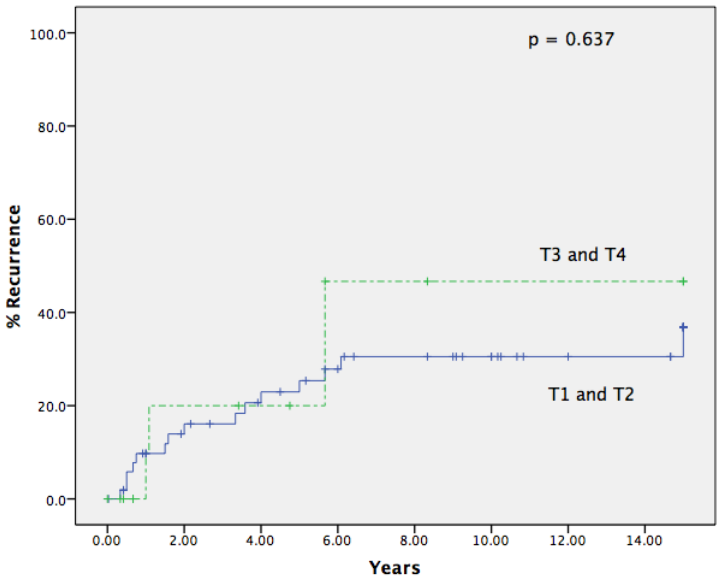

Figure 3.15: Kaplan-Meier recurrence plot for breast cancer patients according to tumour size.

Patients were divided into two groups; a) patients with small tumours,T1 and T2 combined; b) patients with large tumours, T3 and T4 combined. The Kaplan-Meier test showed that the time to recurrence was non-significantly shorter in patients with large tumours (n = 15, 9.71 years, 95% C.I. 5.82 -13.6) when compared with those with small tumours (n = 54, 11.35 years, 95% C.I. 9.53 -12.97, Kaplan-Meier, p = 0.637).

3.3.3.3 Recurrence analysis according to nodal status.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to nodal status (N^{-ve} and N^{+ve}). Patient numbers included in each patient group are displayed in (Table 3.2). The Kaplan-Meier test showed that the mean time to recurrence was significantly shorter in patients with node positive tumours (n = 43, 9.28 years, 95% C.I. 7.03-11.52) when compared with those with node negative tumours (n = 20, 13.83 years, 95% C.I. 12.31 -15.35, p = 0.006, Figure 3.16). Furthermore, Cox Regression (Hazard Ratio) analysis showed that the relative risk of recurrence was 1.8 times more (95% CI 1.58 – 28.49, p = 0.017) in patients with node positive tumours when compared with patients with node negative tumours.

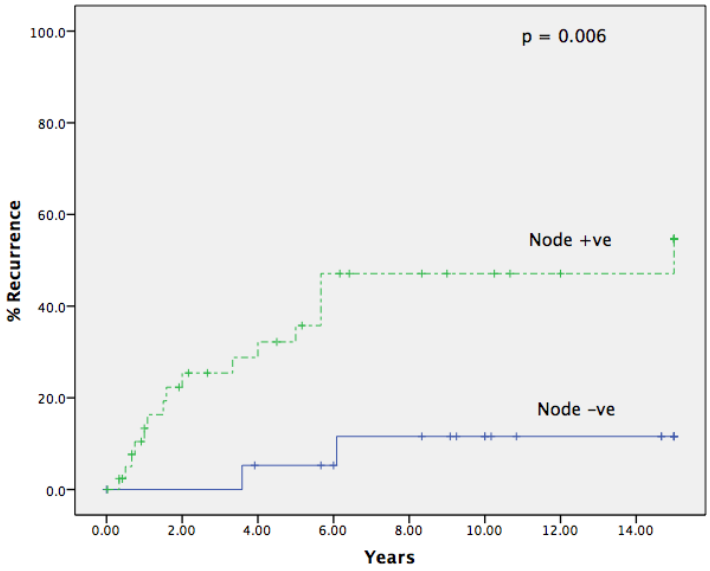

Figure 3.16: Kaplan-Meier recurrence plot for breast cancer patients according to nodal status.

The Kaplan-Meier test showed that the mean time to recurrence was significantly shorter in patients with node positive tumours (n = 43, 9.28 years, 95% C.I. 7.03-11.52) when compared with those with node negative tumours (n = 20, 13.83years, 95% C.I. 12.31 -15.35, p = 0.006).

3.3.3.4 Recurrence analysis according to oestrogen receptor status.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to oestrogen receptor status (ER^{-ve} and ER^{+ve}). Patient numbers included in each patient group are displayed in (Table 3.2). The Kaplan-Meier test showed that the mean time to recurrence was not significantly shorter in patients with ER^{+ve} tumours (n = 32, 9.74 years, 95% C.I. 7.33 – 12.15.) when compared with those with ER^{-ve} tumours (n = 20, 10.68 years, 95% C.I. 7.85 -13.51, p = 0.637, Figure 3.17).

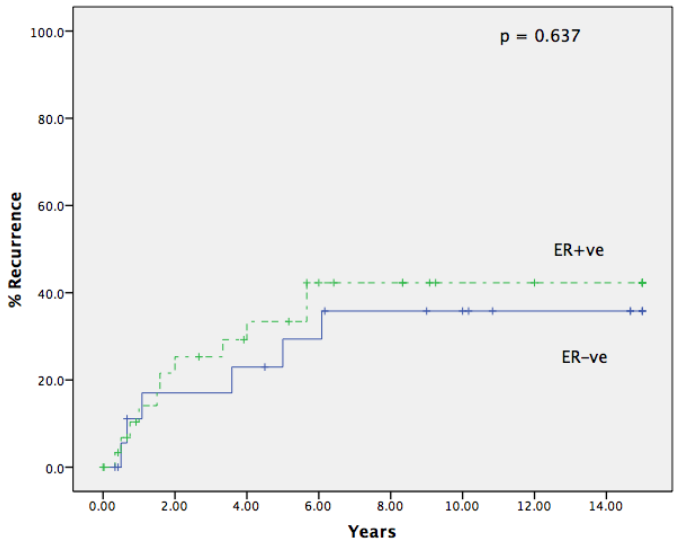

Figure 3.17: Kaplan-Meier recurrence plot for breast cancer patients according to oestrogen receptor status.

The Kaplan-Meier test showed that the mean time to recurrence was not significantly shorter in patients with ER^{+ve} tumours (n = 32, 9.74 years, 95% C.I. 7.33 – 12.15.) when compared with those with ER^{-ve} tumours (n = 20, 10.68 years, 95% C.I. 7.85 -13.51, p = 0.637).

3.3.3.5 Recurrence analysis according to lymphovascular invasion status.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to lymphovasacular invasion status (LV^{-ve} and LV^{+ve}). Patient numbers included in each patient group are displayed in (Table 3.2). The Kaplan-Meier test showed that there was no significant difference in the mean time to recurrence in patients with LV^{-ve} tumours (n = 19, 11.61 years, 95% C.I. 8.73 – 14.49) when compared with those with LV^{+ve} tumours (n = 27, 12.79 years, 95% C.I. 10.59 -14.99, p = 0.771, Figure 3.18).

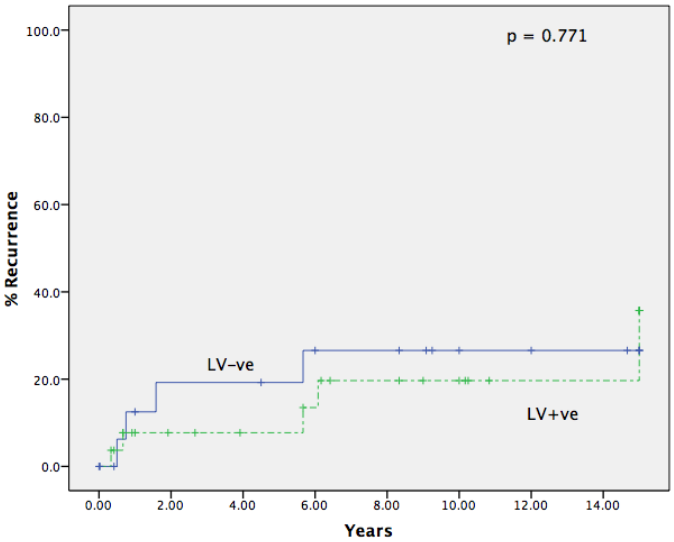

Figure 3.18: Kaplan-Meier recurrence plot for breast cancer patients according to lymphovasacular invasion status.

The Kaplan-Meier test showed that there was no significant difference in the mean time to recurrence in patients with LV^{-ve} tumours (n = 19, 11.61 years, 95% C.I. 8.73 – 14.49) when compared with those with LV^{+ve} tumours (n = 27, 12.79 years, 95% C.I. 10.59 -14.99, p = 0.771).

3.3.3.6 Recurrence analysis according to the NPI.

The Kaplan-Meier test was used to analyse the recurrence data for the entire patient group according to the Nottingham Prognostic Index (NPI). Patient numbers included in each patient group are displayed in (Table 3.1). The Kaplan-Meier test showed that there was no significant difference in the time to recurrence when patients were categorised according to NPI (Figure 3.19, p = 0.604). The mean time to recurrence was (11.14 years, 95% C.I. 8.0 -14.27) in patients with high NPI (NPI >5.4) (n = 19) and (10.58 years, 95% C.I. 8.46-12.7) in those with moderate NPI (NPI 3.4 - 5.4) (n = 37). The mean time to recurrence could not be calculated in patients with low NPI (NPI < 3.4) because of the small number of patients and lack of complete (15 years) follow up data.

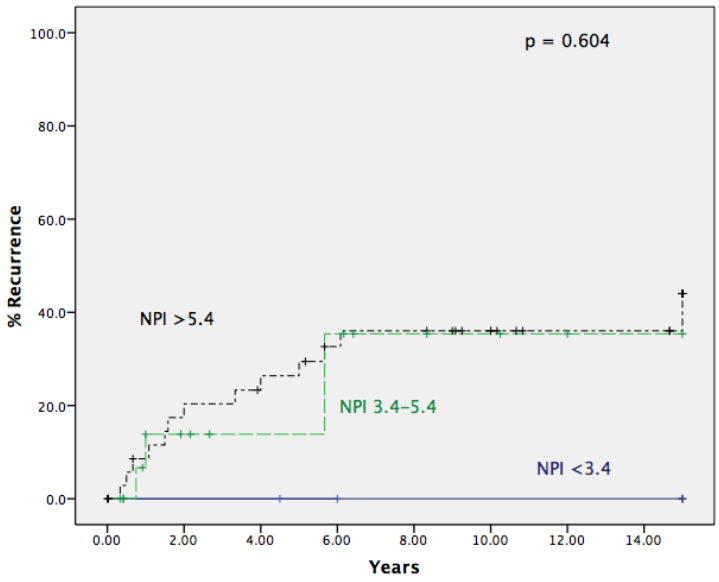

Figure 3.19: Kaplan-Meier recurrence plot for breast cancer patients according to the NPI.

The Kaplan-Meier test showed that there was no significant difference in the time to recurrence in patients with high NPI (NPI >5.4) (n = 19, 11.14 years, 95% C.I. 8.0 -14.27) when compared with those with moderate NPI (NPI 3.4 - 5.4) (n = 36, 10.58 years, 95% C.I. 8.46-12.7; p = 0.604).

3.4 The relative transcriptional expression levels of SIRT1 in breast biopsies

3.4.1 The relative transcriptional expression levels of SIRT1 and patients age.

The relative transcriptional expression levels of SIRT1 gene were determined in (25) normal, (76) non-malignant and (72) malignant biopsies (Table 3.3).

Table 3.3 Patient biopsies used in the SIRT1 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT1 Expression	Normal	25	96.20%	1	3.80%	26	100.00%
	Non-Malignant	76	97.40%	2	2.60%	78	100.00%
	Malignant	72	98.60%	1	1.40%	73	100.00%

The correlation between the relative level of SIRT1 gene expression and patient's age was investigated in a patient group that combined both normal and non-malignant patients. This group of patient represents "normal" breast tissue that derives from a wide range of patient's ages with a normal distribution (Figure 3.20a). The median age of this patient group was 51years (20 years - 84 years). These data showed that there was a marginally significant correlation between increasing chronological age and decreasing relative transcriptional levels of SIRT1 (Spearman's rank correlation coefficient; p = 0.059; Figure 3.20b).

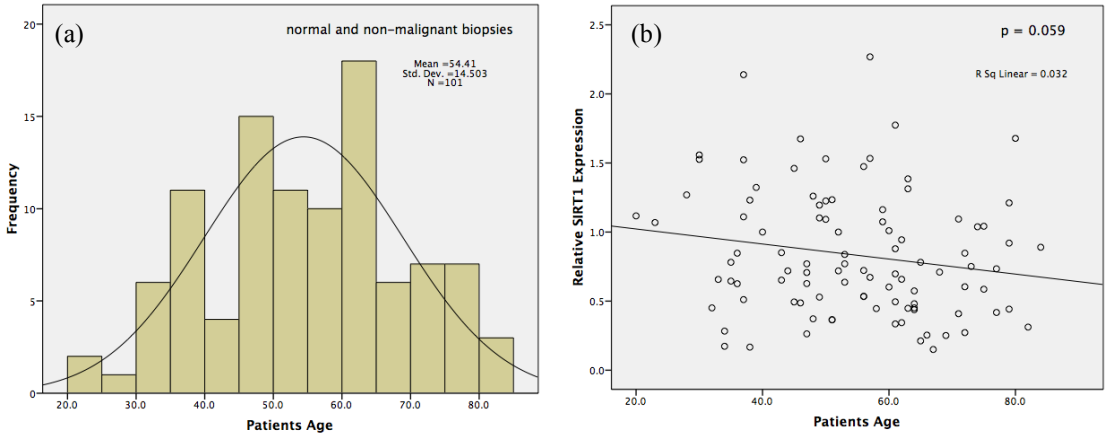

Figure 3.20: (a) Distribution of patient's age in a patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT1 and patient's age in a patient group that combines both normal and non-malignant patients (Spearman test, p = 0.059).

3.4.2 Data distribution of the relative transcriptional expression levels of SIRT1.

The distribution of the relative transcriptional gene expression of SIRT1 was investigated in the three different studied biopsy groups, (Figure 3. 21). The histograms of the distribution of the relative transcriptional gene expression of SIRT1 showed that the range of the SIRT1 gene expression values was not normally distributed; instead the SIRT1 gene expression distribution was positively skewed in all patient groups (Figure 3. 21). Therefore, non-parametric statistical tests were used to analyse all the data for the relative transcriptional gene expression of SIRT1.

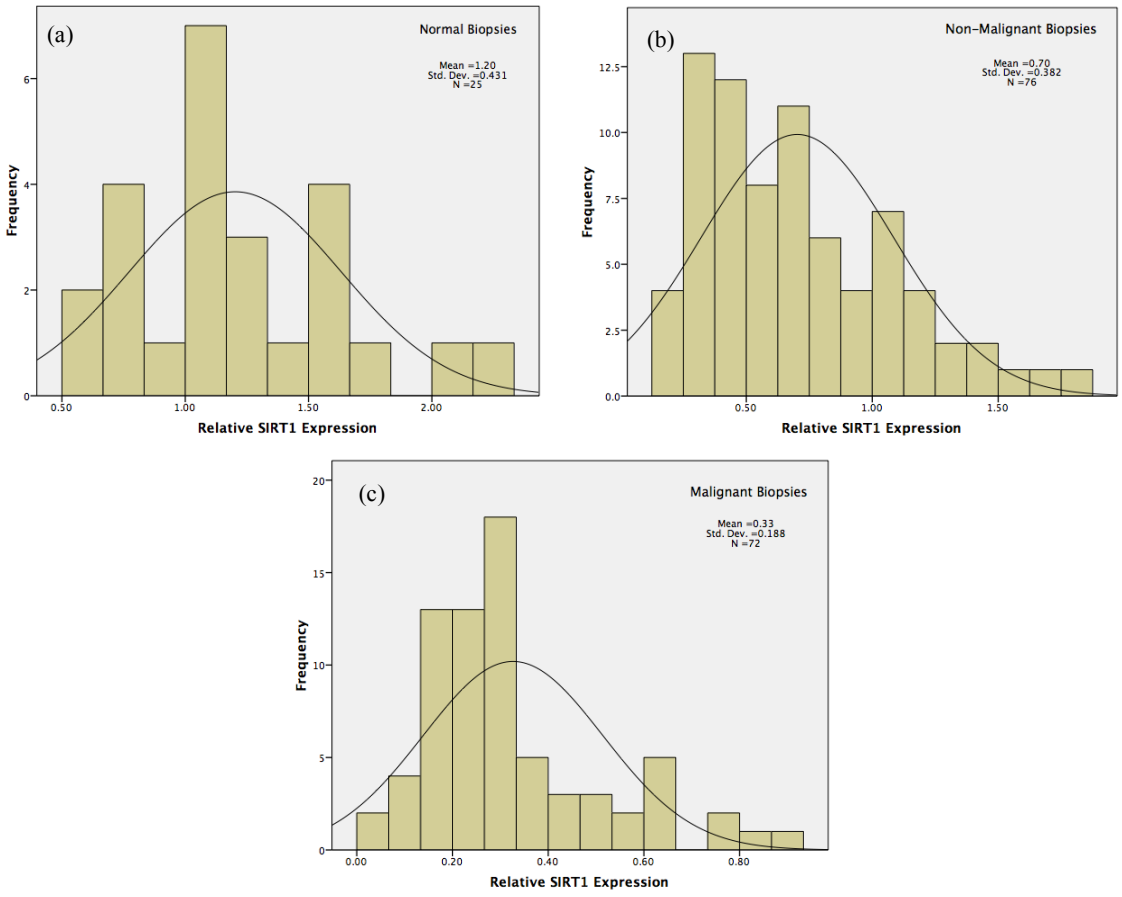

Figure 3.21: Distribution of the relative transcriptional gene expression of SIRT1 in three different patient groups.

(a) Relative transcriptional gene expression of SIRT1 in normal breast biopsies, (b) Relative transcriptional gene expression of SIRT1 in non-malignant breast biopsies, (c) Relative transcriptional gene expression of SIRT1 in malignant breast biopsies.

3.4.3 Comparison of the relative transcriptional expression levels of SIRT1 between patient groups.

The level of SIRT1 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT1 was significantly different between the three groups of breast biopsies ($p<0.001$, Figure 3. 22). This experiment showed that malignant samples had significantly lower levels of SIRT1 gene expression compared to both non-malignant and normal samples ($p<0.001$, Figure 3. 22).

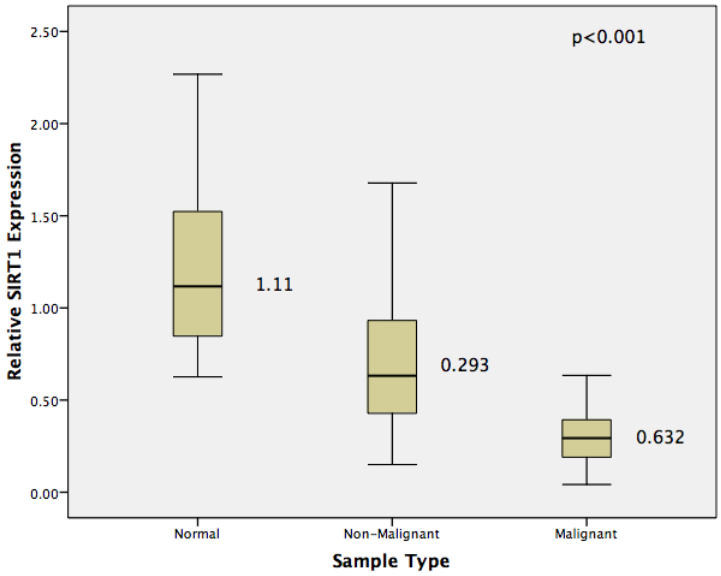

Figure 3.22. Box plot showing the transcriptional expression of SIRT1 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

The median value for each patient group is shown in the clear box to the right of each box plot, ($p < 0.001$).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6.) showed that all three groups differed significantly in SIRT1 gene expression: Malignant versus non-malignant $p < 0.001$, malignant versus normal; $p < 0.001$ and malignant versus non-malignant $p < 0.001$.

3.4.4 Comparison of the relative transcriptional expression levels of SIRT1 according to histopathological markers

The relationships between SIRT1 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.4.4.1 Comparison of the relative transcriptional expression levels of SIRT1 according to tumour grades.

Analysis of the data for the relationship between SIRT1 gene expression and tumour grade showed a significant difference in the relative transcriptional expression levels of SIRT1 between all tumour grades when compared together (Kruskal-Wallis test p = 0.018) (Figure 3. 23). The expression of SIRT1 decreases with increasing severity (higher grade) of tumour.

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6) showed that grade 1 tumours groups differed significantly in SIRT1 gene expression from both grade 2 (p = 0.004) and grade 3 tumours (p = 0.005), whereas there was no significant difference between grade 2 and grade 3 tumours (p = 0.130) (Figure 3. 23).

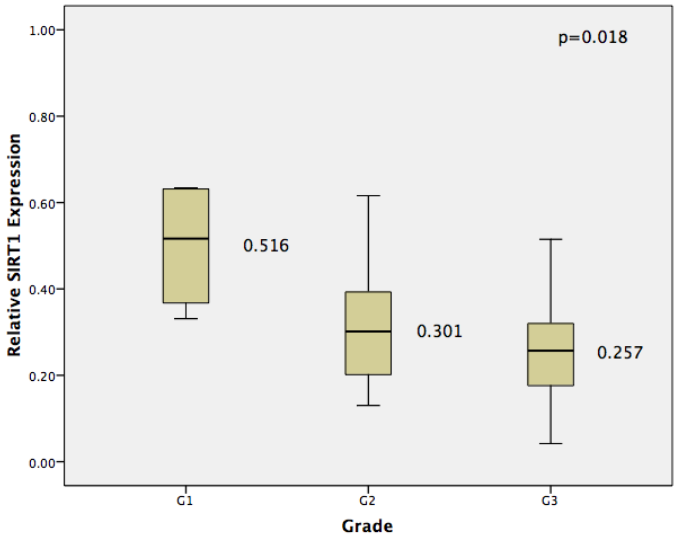

Figure 3.23: Box plot showing the relative transcriptional expression of in breast cancer biopsies by tumour grade.

n = 4 for grade 1, 32 for grade 2 and 29 for grade 3 *(*Kruskal Wallis Test, *p = 0.018)*. The median value for each patient group is shown in the clear box to the right of each box plot.

3.4.4.2 Comparison of the relative transcriptional expression levels of SIRT1 according to tumour size, nodal status, oestrogen receptor status and lymphovascular invasion

Analysis of the data for the relationship between SIRT1 gene expression and tumour size, nodal status, oestrogen receptor status and lymphovascular invasion, showed no significant differences in the relative transcriptional expression levels of SIRT1 between any

categories when compared together (Figure 3. 24 a, b, c, and d). Table 3.6 shows the Kruskal-Wallis or Mann Whitney p values for all comparisons.

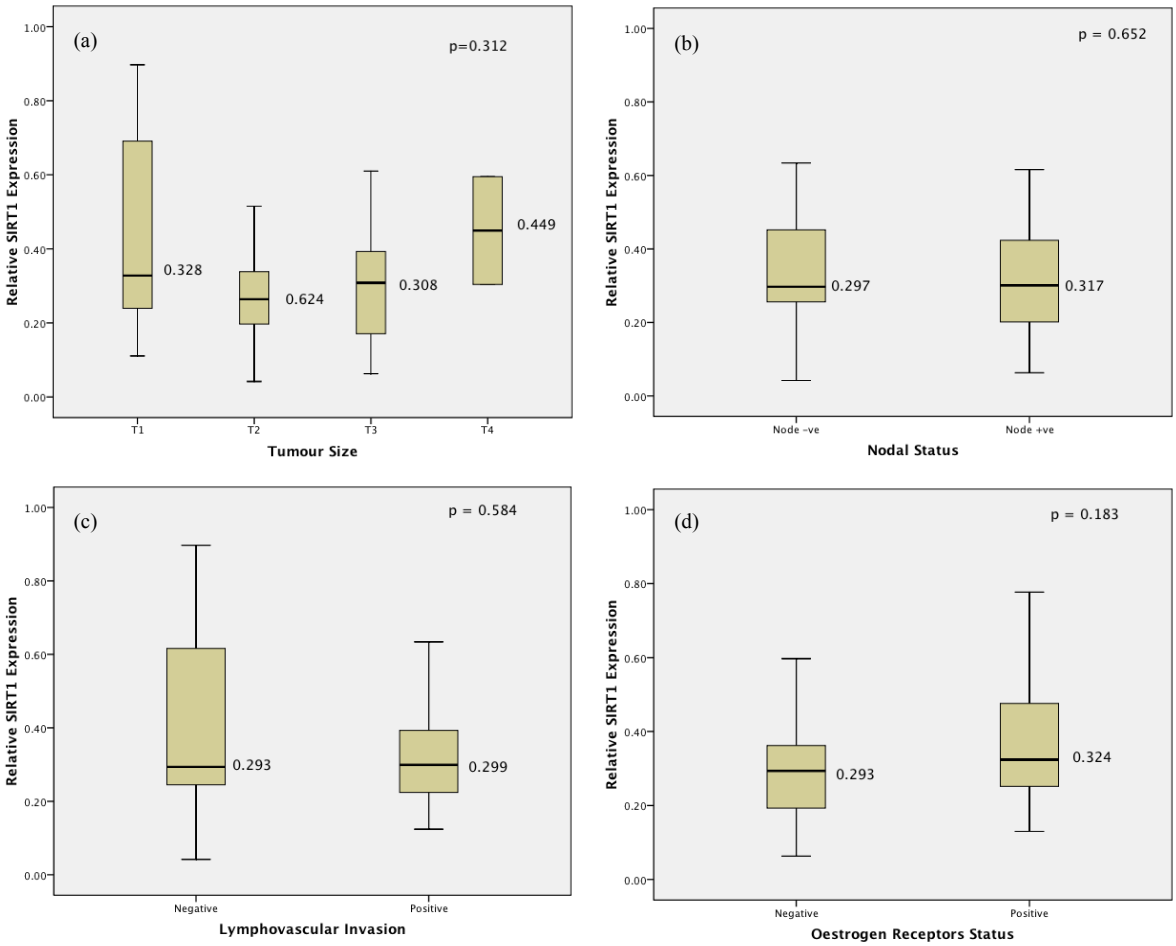

Figure 3.24: Box plots showing the relative transcriptional expression of SIRT1 in breast cancer biopsies by known prognostic markers:

(a) tumour size, n = 11 for T1, 44 for T2, 14 for T3 and 2 for T4 *(*Kruskal Wallis Test, *p=0.312)* (b) nodal status, n = 20 for node –ve and 43 for node +ve Mann Whitney test p = 0.652, (c) oestrogen receptor status, n = 20 for ER^{-ve} and 32 for ER^{+ve} (Mann Whitney test p = 0.183. (d) lymphovascular invasion (LVI) n = 18 for LV^{-ve} and 28 for LV^{+ve} (Mann Whitney test p = 0.584). The median value for each patient group is shown right of each box plot.

3.4.5 The relationship of the relative transcriptional expression levels of SIRT1 in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT1 relative gene expression, the patients were grouped in tertiles according to the level of SIRT1 gene expression. In analyses shown below, the two tertiles with lower SIRT1 gene expression have been combined and are compared to the highest level of SIRT1 gene expression tertile group. Table 3.4 and Table 3.5 show the numbers of patients included in each group.

Importantly, this data can be influenced by two key parameters:

- Some patients have clinical criteria that give a very poor prognosis and such patients are likely to die before any differences, due to genetic influences, are detectable.

- Patients with clinically accepted very good prognosis are unlikely to provide useful data.

The data, therefore, were also analysed after focusing on that patient group with an intermediate clinical prognosis, identified using the NPI (Material and Methods) Specifically, patients with NPI values between 3.4 and 5.4 were included in this analysis.

In addition, patients were classified for analysis by oestrogen receptor status, because this is considered to be an important clinically prognostic factor, but this is not included in the Nottingham Prognostic Index.

3.4.5.1 SIRT1 and survival.

3.4.5.1.1 Survival analysis in entire patient cohort according to SIRT1 levels.

Analysis of the survival data for the entire patient group (Table 3.4) using the Kaplan-Meier test showed that the survival period was non-significantly shorter in patients with tumours that express high levels of SIRT1 (n = 24, 9.83 years, 95% C.I. 7.6 -12.06) when compared to those whose tumours express low levels of SIRT1 (n = 45, 11.47 years, 95% C.I. 9.77 -13.17, Kaplan-Meier p = 0.235; Figure 3. 25).

Table 3.4: Number of breast cancer patient and cancer specific deaths of all patient groups included in the Kaplan-Meier survival analysis.

SIRT1 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT1 gene expression	45	12	25	5	18	5
High SIRT1 gene expression	24	9	11	5	14	5
Overall	69	21	36	10	32	17

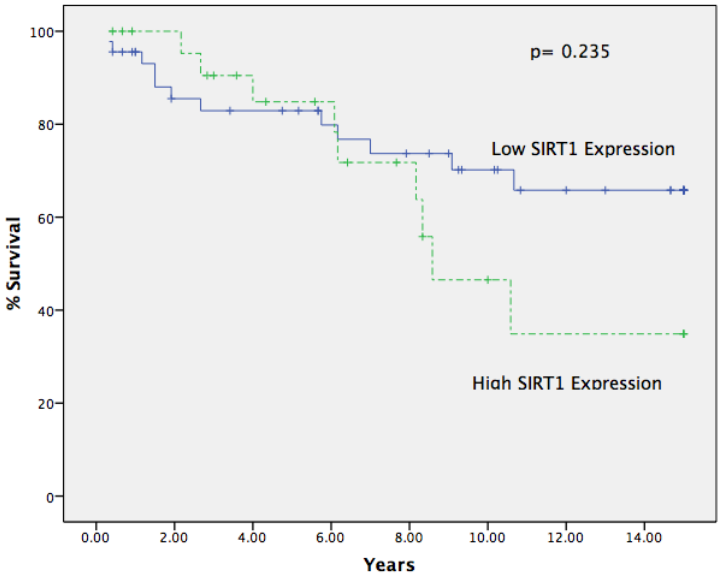

Figure 3.25: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT1 expression.

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a non significant decrease in the survival period for patients with tumours that express high levels SIRT1 (n=24, 9.83 years, 95% C.I. 7.6 -12.06) when compared to those whose tumours express low levels of SIRT1 (n=45, 11.47 years, 95% C.I. 9.77 -13.17, Kaplan-Meier p=0.235).

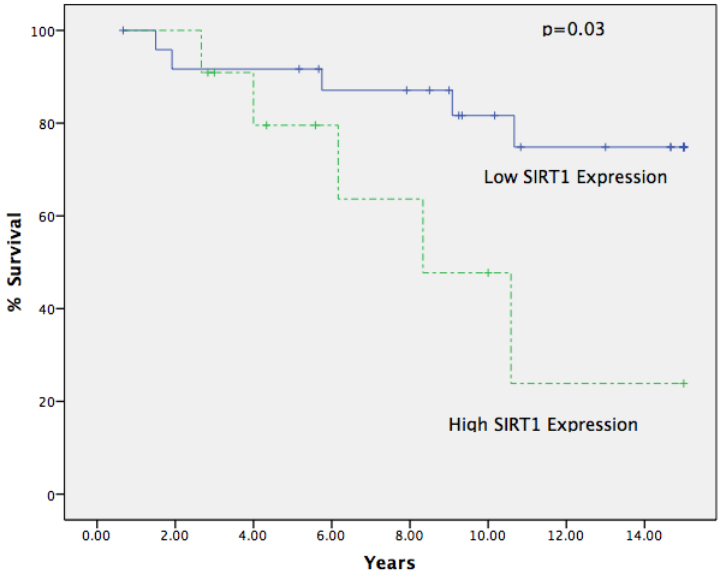

Figure 3.26: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT1 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a significant decrease in the survival period for patients with tumours that express high levels SIRT1 (n = 11, 9.10 years, 95% C.I. 6.6-12.15) when compared to those whose tumours express low levels of SIRT1 (n = 25, 12.85 years, 95% C.I. 11.11 -14.58, Kaplan-Meier p = 0.03).

3.4.5.1.2 Survival analysis in NPI selected patients according to SIRT1 levels.

Analysis of the survival data in patients with moderately good prognosis (NPI between 3.4-5.4, Table 3.4) demonstrated that survival period was significantly shorter in patients with tumours that expressed high levels of SIRT1 (n = 11, 9.10 years, 95% C.I. 6.6-12.15) when compared to those whose tumours express low levels of SIRT1 (n = 25, 12.85 years, 95% C.I. 11.11 -14.58, Kaplan-Meier p = 0.03; Figure 3. 26).

3.4.5.1.3 Survival analysis in ER+ve selected patients according to SIRT1 levels.

Analysis of the cancer specific survival data in patients with oestrogen receptor positive tumours (ER^{+ve}) tumours did not show any significant differences in the survival period between patients with tumours that expressed high levels of SIRT1 (n = 14, 9.46 years, 95% C.I. 6.35-12.57) when compared to those whose tumours express low levels of SIRT1 (n = 18, 8.13years, 95% C.I% 8.13-13.88, Kaplan-Meier p = 0.452; Figure 3.27).

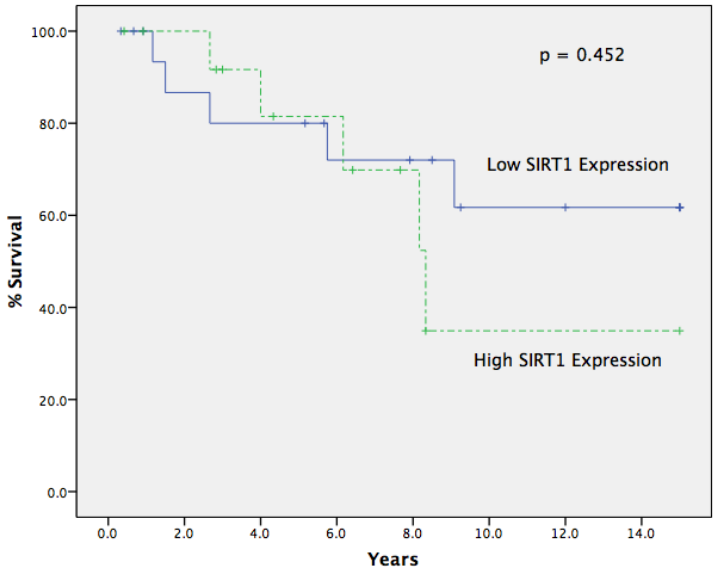

Figure 3.27: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT1 expression in patients with ER+ve.

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows no significant difference in the survival period for patients with tumours that express high levels SIRT1 (n = 14, 9.46 years, 95% C.I. 6.35-12.57) when compared to those whose tumours express low levels of SIRT1 (n = 18, 8.13years, 95% C.I% 8.13-13.88, Kaplan-Meier p = 0.452.

3.4.5.2 SIRT1 and recurrence.

3.4.5.2.1 Recurrence analysis in entire patient cohort according to SIRT1 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, and showed a clear but, statistically non-significant trend towards shorter recurrence interval in patients with high levels of SIRT1 gene expression when all patients were included. Patient numbers are shown in (Table 3.5). Time to recurrence was shorter in patients with tumours that expressed high levels of SIRT1 (n = 23, 8.98 years, 95% C.I. 6.16-11.72), when compared to those whose tumours expressed low levels of SIRT1 (n = 46, 12.08 years, 95% C.I. 10.30 -13.86, Kaplan-Meier p = 0.08; Figure 3. 28).

Table 3.5: Number of breast cancer patient and tumour recurrences of all patient groups included in the Kaplan-Meier survival analysis.

SIRT1 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT1 gene expression	45	10	26	7	12	6
High SIRT1 gene expression	24	9	11	6	20	5
Overall	69	21	37	13	32	11

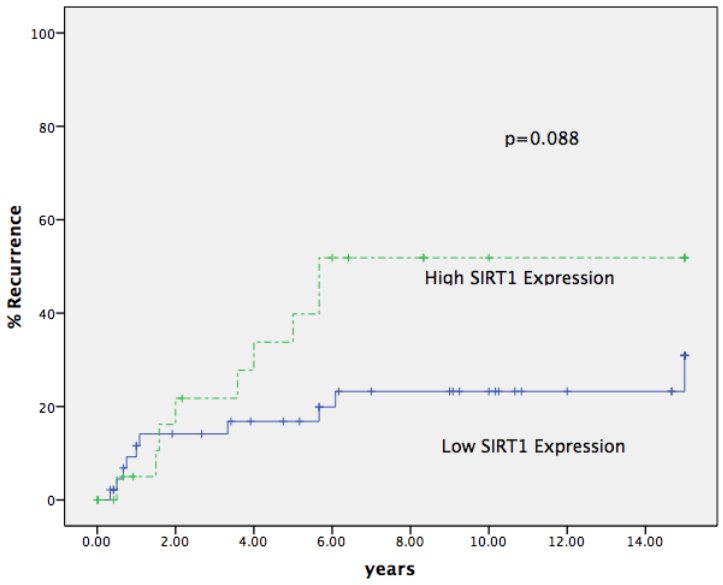

Figure 3.28: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT1 expression.

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed that time to recurrence was shorter in patients with tumours that expressed high levels of SIRT1 (n = 23, 8.98 years, 95% C.I. 6.16-11.72) when compared to those whose tumours express low levels of SIRT1 (n = 46, 12.08 years, 95% C.I. 10.30 -13.86, Kaplan-Meier p = 0.08.

3.4.5.2.2 Recurrence analysis in NPI selected patients according to SIRT1 levels.

When tumour recurrence among patients with moderately good prognosis (NPI between 3.4-5.4) was analysed (Table 3.5), there was a significant difference according to the levels of SIRT1 gene expression. The Kaplan-Meier recurrence analysis showed that time to recurrence was significantly shorter in patients with tumours that expressed high levels of SIRT1 (n = 11, 6.62 years, 2.66-10.58) when compared to those whose tumours expressed low levels of SIRT1 (n = 26, 12.02 years, 95% C.I. 9.75 -14.29, Kaplan-Meier p= 0.024; Figure 3.29).

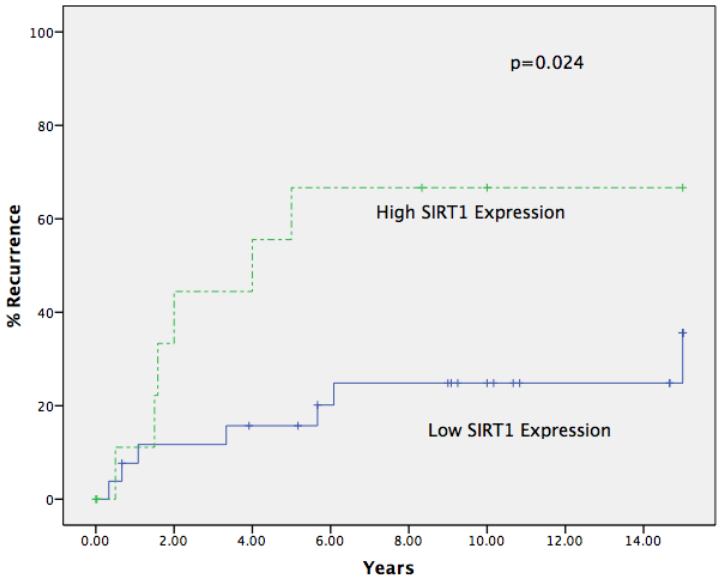

Figure 3.29: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT1 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed that time to recurrence was significantly shorter in patients with tumours that expressed high levels of SIRT1 (n = 11, 6.62 years, 95% C.I. 2.66-10.58) when compared to those whose tumours express low levels of SIRT1 (n = 26, 12.02 years, 95% C.I. 9.75 -14.29, Kaplan-Meier p = 0.025.

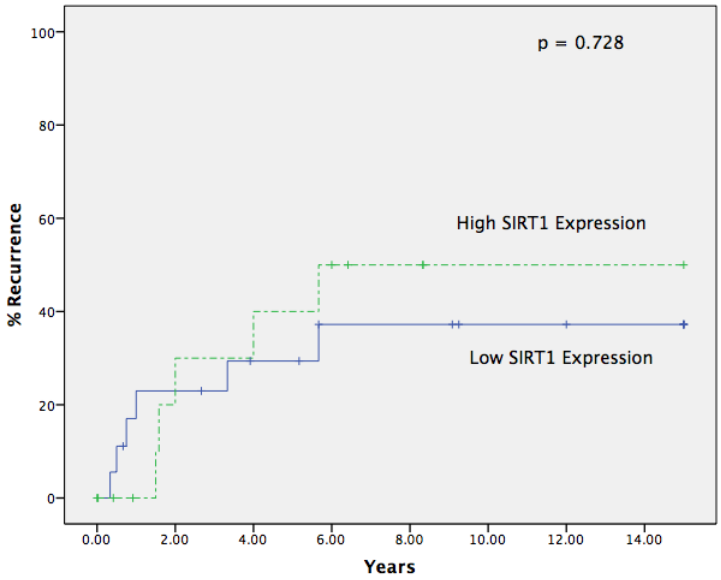

Figure 3.30: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT1 expression in patient with ER^{+ve} tumours.

High and low SIRT1 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed no significant difference in time to recurrence between patients with tumours that expressed high levels of SIRT1 (n = 14, 8.97 years, 95% C.I. 5.17-12.77) when compared to those whose tumours express low levels of SIRT1 (n = 18, 10.22 years, 95% C.I. 7.13-13.31, Kaplan-Meier p = 0.728).

3.4.5.2.3 Recurrence analysis in ER $^{+ve}$ patients according to SIRT1 levels.

Further analysis of recurrence within the subgroup of patients with ER^{+ve} tumours (Table 3.5) failed to reveal any significant difference in the time to recurrence between patients with tumours that expressed high levels of SIRT1 (n = 14, 8.97 years, 95% C.I. 5.17-12.77) when compared to those whose tumours expressed low levels of SIRT1 (n = 18, 10.22 years, 95% C.I. 7.13-13.31, Kaplan-Meier p = 0.728; Figure 3. 30).

Table 3.6: p values for comparisons of the relative transcriptional expression of all sirtuin genes in the three patient groups and in the malignant biopsies according to the known pathological markers.

Gene	Biopsy type				Grade				Size	Node
	Overall	N-NM	N-M	NM-M	Overall	1~2	1~3	2~3	Overall	
SIRT1	0.001	0.001	0.001	0.001	0.018	0.044	0.005	0.13	0.312	0.652
SIRT2	0.001	0.704	0.001	0.001	0.086	0.033	0.057	0.417	0.495	0.592
SIRT3	0.001	0.301	0.001	0.001	0.049	0.717	0.217	0.022	0.312	0.305
SIRT4	0.001	0.001	0.729	0.001	0.875	0.846	0.669	0.723	0.648	0.059
SIRT5	0.001	0.001	0.001	0.072	0.973	0.978	0.714	0.971	0.162	0.508
SIRT6	0.001	0.019	0.001	0.001	0.002	0.61	0.03	0.001	0.478	0.697
SIRT7	0.001	0.001	0.001	0.51	0.075	0.048	0.062	0.237	0.678	0.763

Gene	ER	LV	NPI	Survival			Recurrence		
				overall	NPI	ER+ve	overall	NPI	ER+ve
SIRT1	0.183	0.584	0.143	0.235	0.03	0.452	0.088	0.024	0.728
SIRT2	0.445	0.933	0.237	0.529	0.619	0.73	0.947	0.764	0.352
SIRT3	0.216	0.662	0.782	0.789	0.968	0.487	0.866	0.903	0.519
SIRT4	0.347	0.473	0.464	0.38	0.384	0.135	0.098	0.125	0.023
SIRT5	0.335	0.242	0.57	0.305	0.983	0.538	0.272	0.287	0.531
SIRT6	0.508	0.392	0.309	0.009	0.004	0.002	0.178	0.067	0.282
SIRT7	0.486	0.317	0.21	0.05	0.1	0.045	0.677	0.994	0.684

3.4.6 Conclusions.

3.4.6.1 Breast biopsies and the characteristics of patient cohorts.

The relative transcriptional expression levels of sirtuin genes were determined in a sample of archival breast biopsies; normal (n = 25), non-malignant (n = 78) and malignant (n = 73) breast biopsies.

Patient's age was normally distributed in all patient groups. Patient's age was significantly different in normal when compared with patient's age in both non-malignant and malignant groups.

The Kaplan-Meier analysis for cancer specific survival according to the known prognostic markers (Table 3.1) was only significant when breast cancer patients were grouped according to nodal status ($p = 0.003$) and according to the NPI ($p = 0.015$). Tumour size showed significant association with survival only when patients were grouped, according to the tumour size, into patients with small tumours (T1 and T2) and patients with large tumours (T3 and T4) ($p = 0.013$).

The Kaplan-Meier analysis for breast cancer recurrence according to the known prognostic markers was only significant (Table 3.2) when breast cancer patients were grouped according to nodal status ($p = 0.006$).

3.4.6.2 The relative transcriptional expression levels of sirtuin 1 in breast biopsies.

This study investigated the relative transcriptional expression levels of the SIRT1 gene in (25) normal, (76) non-malignant and (72) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT1, as the data distributions were positively skewed in all patient groups.

The analysis of the relative transcriptional expression of SIRT1 with age in " normal patients" (normal and non-malignant patients) showed marginally significant correlation between increasing chronological age and decreasing the relative transcriptional levels of SIRT1 ($p = 0.059$).

The data for SIRT1 gene expression variation between breast cancer biopsies showed that there were significant differences in SIRT1 gene expression between biopsies from malignant, non-malignant and normal patient groups. The expression level for SIRT1 is significantly lower, in malignant compared to both non-malignant and normal patient groups. SIRT1 gene expression also differs significantly between the non-malignant and normal patient groups, which differ in their age distributions, even when corrected for age (Table 3.6).

The relative transcriptional expression level of SIRT1 was only significantly associated with tumour grade when breast cancer patients were divided according to the known histopathological markers. There was a significant correlation between decreased SIRT1 gene expression and higher tumour grade.

The Kaplan-Meier analysis for cancer specific survival and tumour recurrence according to relative transcriptional expression level of SIRT1, showed no significant association between SIRT1 levels and survival period or time to recurrence when all patients were included. However, analysis of the data showed clearly that the survival curves, according to SIRT1 levels, dissociate after 5 years. Usually, breast cancer patients with aggressive tumours experience recurrence or metastasis in the first two years. Those patients with such a poor prognosis can be readily evaluated using the commonly used criterion of an NPI > 5.4. Therefore, further analysis of the breast cancer specific survival and time to recurrence data, according to relative transcriptional expression level of SIRT1, was performed in a patient group selected as having an intermediate clinical prognosis using the NPI (NPI between 3.4 and 5.4). This analysis showed that lower SIRT1 gene expression is prognostic of better long-term patient survival and lower tumour recurrence in a patient group selected by NPI to have an intermediate clinical prognosis.

3.2 The relative transcriptional expression levels of SIRT2 in breast biopsies.

3.2.1 The relative transcriptional expression levels of SIRT2 gene and patients age.

The relative transcriptional expression levels of SIRT2 gene were determined in 24 normal biopsies, 75 non-malignant and 73 malignant patient biopsies (Table 3.7).

Table 3.7 Patient biopsies used in the SIRT2 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT2 Expression	Normal	24	92.30%	2	7.70%	26	100.00%
	Non-Malignant	75	96.20%	3	3.80%	78	100.00%
	Malignant	73	100.00%	0	0.00%	73	100.00%

The correlation between the relative level of SIRT2 gene expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3. 31a). The median age of this patient group was 51 years (20 years – 84 years). This data showed that there was no significant association between the relative transcription levels of SIRT2 and chronological age (Spearman's rank correlation coefficient; p = 0.173; Figure 3. 32b).

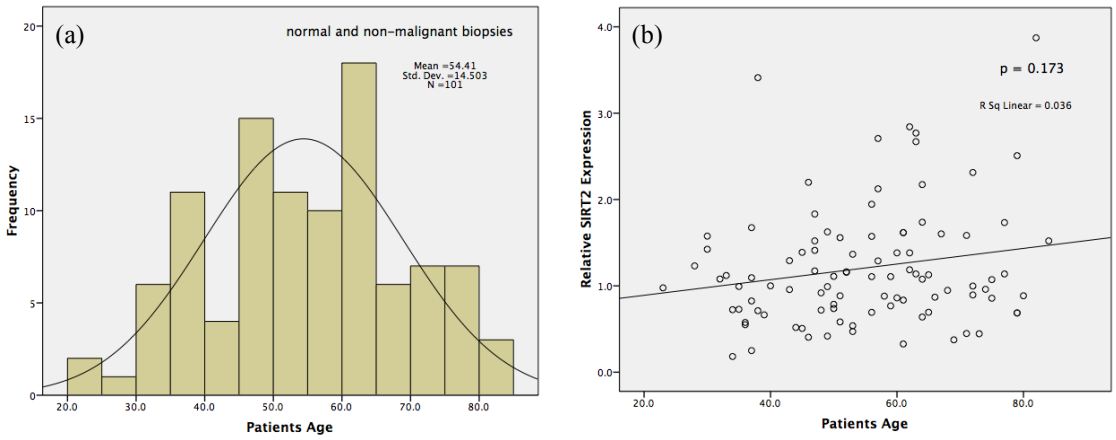

Figure 3.31: (a) Distribution of patient's age in patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT2 and patient's age in patient group that combines both normal and non-malignant patients (spearman test, p = 0.173).

3.2.2 Data distribution of the relative transcriptional expression levels of SIRT2.

The distribution of the relative transcriptional gene expression of SIRT2 was investigated in the three different studied biopsy types (Figure 3. 32). The histograms of the distribution of the relative transcriptional gene expression of SIRT2 showed that the range of the SIRT2 gene expression values was not normally distributed; instead the SIRT2 gene expression distribution was positive skewed in all groups (Figure 3.32). Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT2.

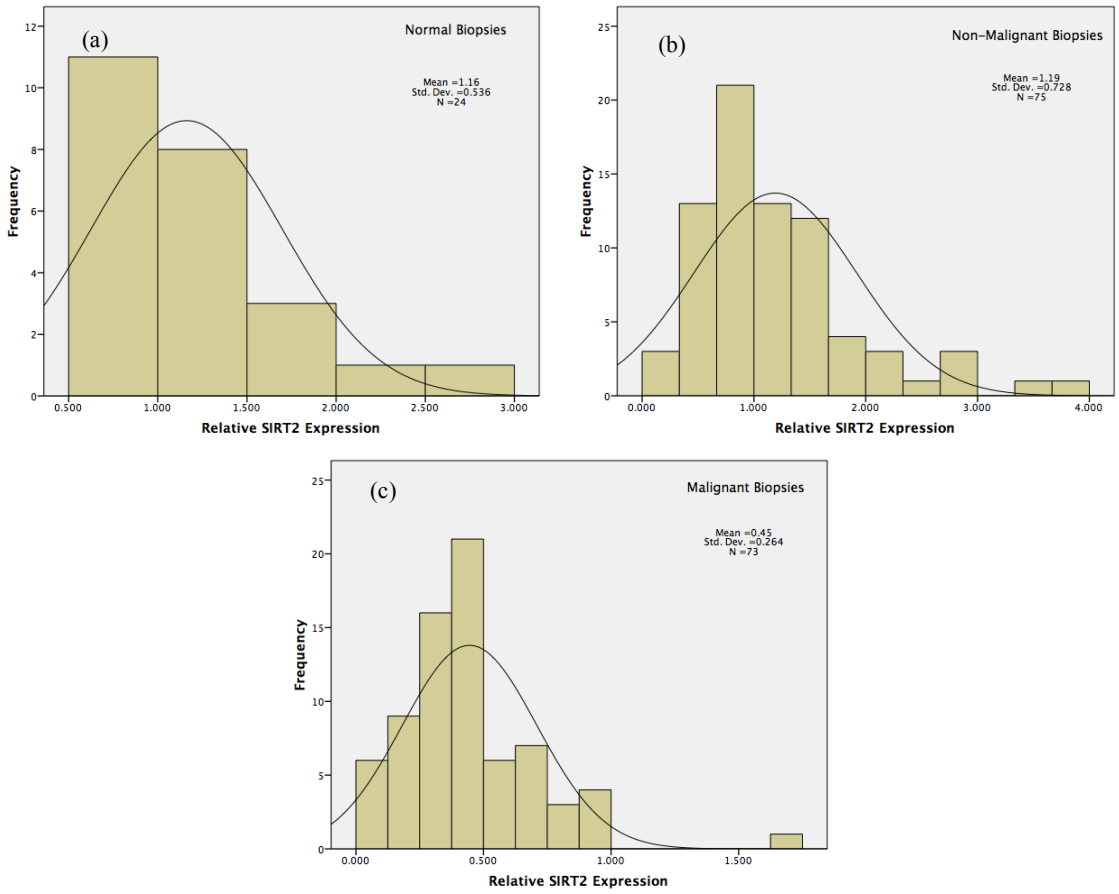

Figure 3.32: Distribution of the relative transcriptional gene expression of SIRT2 in three different groups.

Relative transcriptional gene expression of SIRT2 in normal breast biopsies, (b) Relative transcriptional gene expression of SIRT2 in non-malignant breast biopsies, (c) Relative transcriptional gene expression of SIRT2 in malignant breast biopsies.

3.2.3 Comparison of the relative transcriptional expression levels of SIRT2 between patient groups.

The level of SIRT2 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT2 was significantly different between the three groups of breast biopsies ($p < 0.001$, Figure 3. 33). This experiment showed that malignant samples have significantly lower levels of SIRT2 gene expression compared to both non-malignant and normal samples ($p < 0.001$, Figure 3.33).

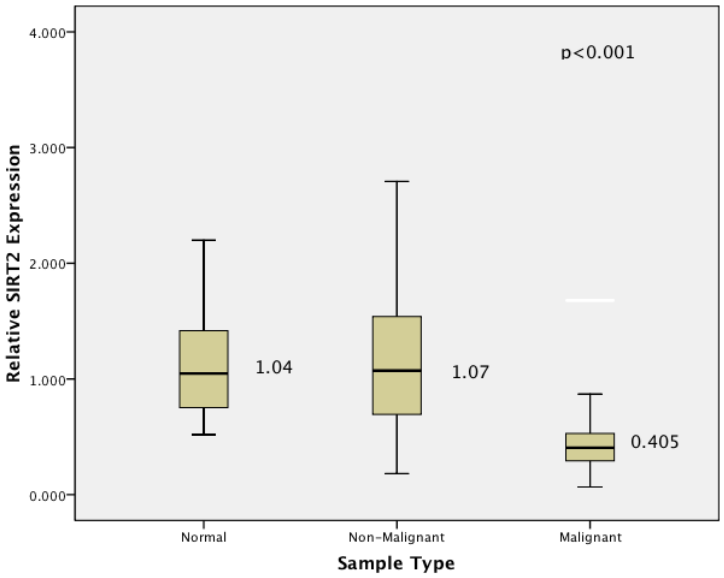

Figure 3.33: Box plot showing the transcriptional expression of SIRT2 in normal, non-malignant and malignant breast biopsies, relative to *HPRT*.

The median value for each patient group is shown to the right of each box plot (p<0.001).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3. 6) showed that all the malignant groups differed significantly in SIRT2 gene expression from both non-malignant and normal groups (malignant versus non-malignant $p < 0.001$, malignant versus normal; $p < 0.001$). However, there was no significant difference between the malignant and non-malignant groups ($p < 0.704$).

3.2.4 Comparison of the relative transcriptional expression levels of SIRT2 according to histopathological markers.

The relationships between SIRT2 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.2.4.1 Comparison of the relative transcriptional expression levels of SIRT2 according to tumour grades.

Analysis of the data for the relationship between SIRT2 gene expression and tumour grade showed a trend toward significance when the levels of SIRT2 gene expression were compared between all three tumour grades together (Kruskal-Wallis test p = 0.086) (Fig 3.34).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3. 6) showed that grade 1 tumour groups differed significantly in SIRT2 gene expression from both grade 2 (p = 0.033) and grade 3 tumours (p = 0.057). The expression of SIRT2 decreased with increasing severity (higher grade) of tumour. There was no significant difference between grade 2 and grade 3 tumours (p = 0.417; Figure 3.34).

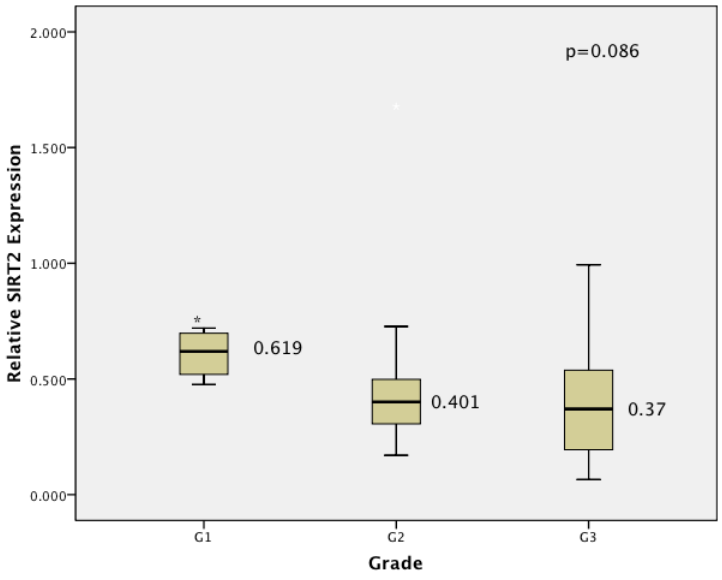

Figure 3.34: Box plot showing the relative transcriptional expression of in breast cancer biopsies by tumour grade.

n = 4 for grade 1, n = 32 for grade 2 and n = 31 for grade 3 *(*Kruskal Wallis Test, *p = 0.089)*. The median value for each patient group is shown to the right of each box plot.

3.2.4.2 Comparison of the relative transcriptional expression levels of SIRT2 according to tumour size, nodal status, oestrogen receptor status and lymphovascular invasion.

Analysis of the data for the relationship between SIRT2 gene expression and tumour size, nodal status, oestrogen receptor status and lymphovascular invasion showed no significant differences in the relative transcriptional expression levels of SIRT2 between any categories when compared together (Figure 3.35 a, b, c, and d). Table 3.6 shows the Kruskal-Wallis test p values for all comparisons.

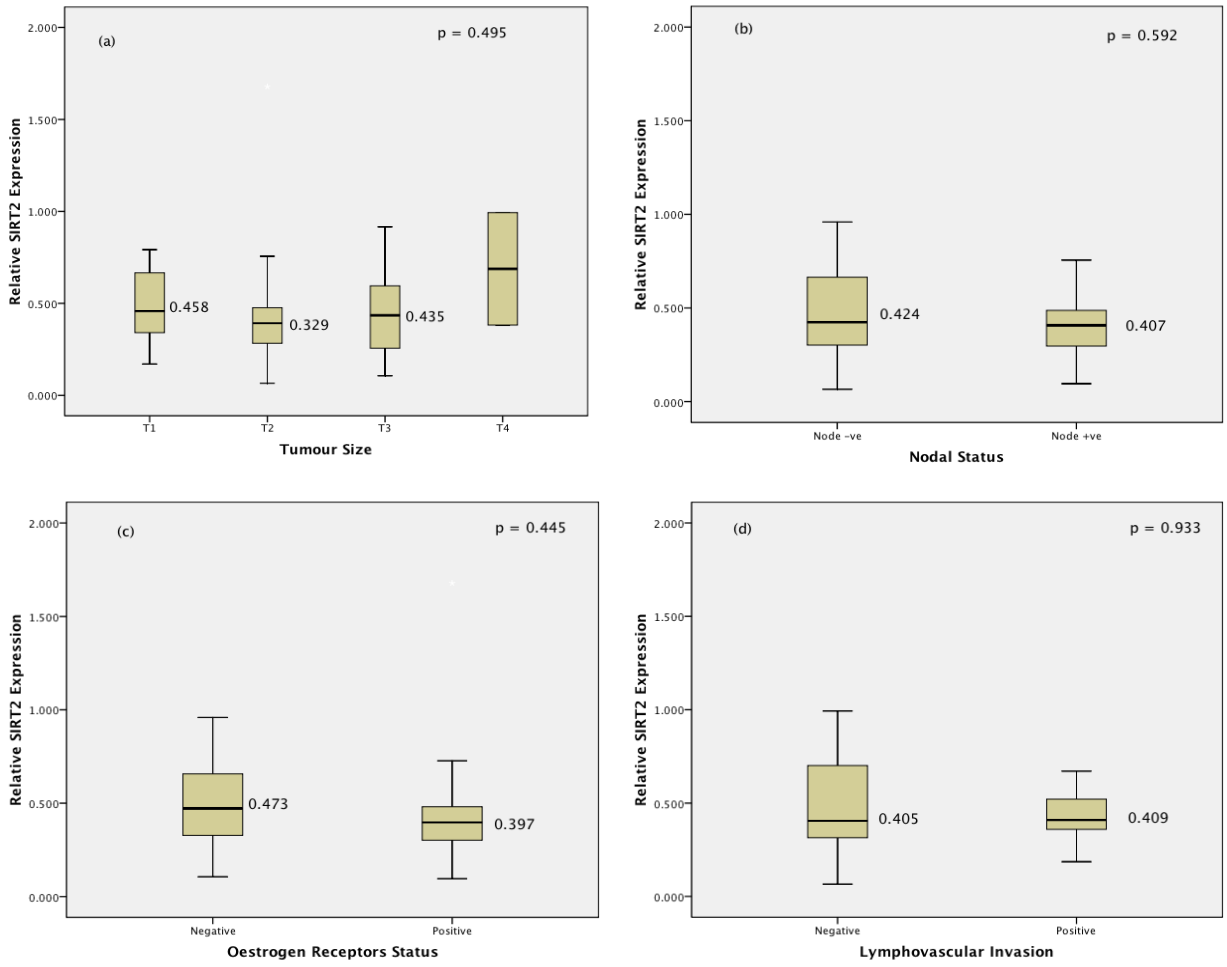

Figure 3.35: Box plots showing the relative transcriptional expression of SIRT2 in breast cancer biopsies by:

(a) Tumour size, n = 11 for T1, n = 45 for T2 ,n = 14 for T3 and 2for T4 (Kruskal Wallis Test, p=0.495). (b) Nodal status, n = 20 for node –ve and n = 44 for node +ve (Mann Whitney test p = 0.592). (c) Oestrogen receptor status, n = 21 for ER^{-ve} and n =32 for ER^{+ve} (Mann Whitney test p = 0.445). d) Lymphovascular invasion (LV) n = 19 for LV^{-ve} and n = 29 for LV^{+ve} (Mann Whitney test p = 0.933). The median value for each patient group is shown right of each box.

3.2.5 The relationship of relative SIRT2 gene expression in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT2 relative gene expression, the patients were grouped in tertiles according to the level of SIRT2 gene expression. In analyses shown below, the two tertiles with higher SIRT2 gene expression levels have been combined and are compared to the lowest level of SIRT2 gene expression tertile group. Table 3.10 and Table 3.11 show the numbers of patients included in each group.

3.2.5.1 SIRT2 and survival.

3.2.5.1.1 Survival analysis in entire patient cohort according to SIRT2 levels.

Analysis of the survival data for the entire patient group using the Kaplan-Meier test showed no significant difference in survival period when all patients are included (Table 3.8). The survival period was non-significantly shorter in patients with tumours that expressed high levels of SIRT2 (n = 48, 10.79 years, 95% C.I. 9.14 -12.44) when compared to those whose tumours expressed low levels of SIRT2 (n = 22, 11.49 years, 95% C.I. 9.08 – 13.09, Kaplan-Meier p = 0.529 (Figure 3. 36).

Table 3.8:Number of breast cancer patient and cancer specific deaths of all breast cancer patients groups included in the Kaplan-Meier survival analysis.

SIRT2 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT2 gene expression	22	6	11	3	9	3
High SIRT2 gene expression	48	15	25	7	22	7
Overall	70	21	36	11	31	10

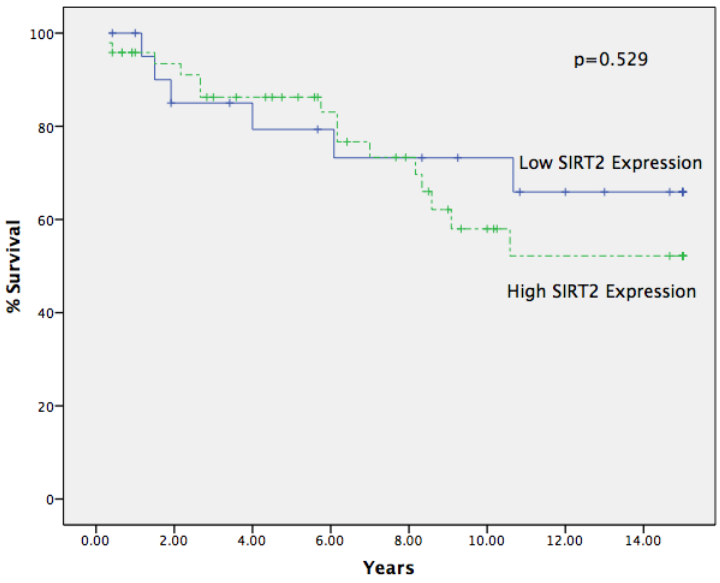

Figure 3.36: Kaplan-Meier Survival plot for breast cancer patients according to the level of *SIRT2* expression.

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows a non significant decrease in the survival period for patients with tumours that express high levels SIRT2 (n = 48, 10.79 years, 95% C.I. 9.14 - 12.44) when compared to those whose tumours express low levels of SIRT2 (n = 22, 11.49 years, 95% C.I. 9.08 – 13.09, Kaplan-Meier p = 0.529.

3.2.5.1.2 Survival analysis in NPI selected patients according to SIRT2 levels.

Analysis of the survival data for the NPI-selected patient group (Table 3. 8) using the Kaplan-Meier test did not show any significant difference in the survival period in patients with tumours that expressed high levels of SIRT2 (n = 25, 11.72 years, 95% C.I. 9.75 - 15.07) when compared to those whose tumours expressed low levels of SIRT2 (n = 11, 12.30 years, 95% C.I. 9.53 – 15.07, Kaplan-Meier p = 0.619; Figure 3. 37).

3.2.5.1.3 Survival analysis ER^{+ve} selected patients according to SIRT2 levels.

Analysis of the survival data for the ER+ve patient group (Table 3.10) using the Kaplan-Meier test showed no significant difference in the survival period in patients with tumours that expressed high levels of SIRT2 (n = 22, 10.74 years, 95% C.I. 6.78-14.7) when compared to those whose tumours expressed low levels of SIRT2 (n = 9, 10.07 years, 95% C.I. 7.58 – 12.57, Kaplan-Meier p = 0.73 Figure 3. 38).

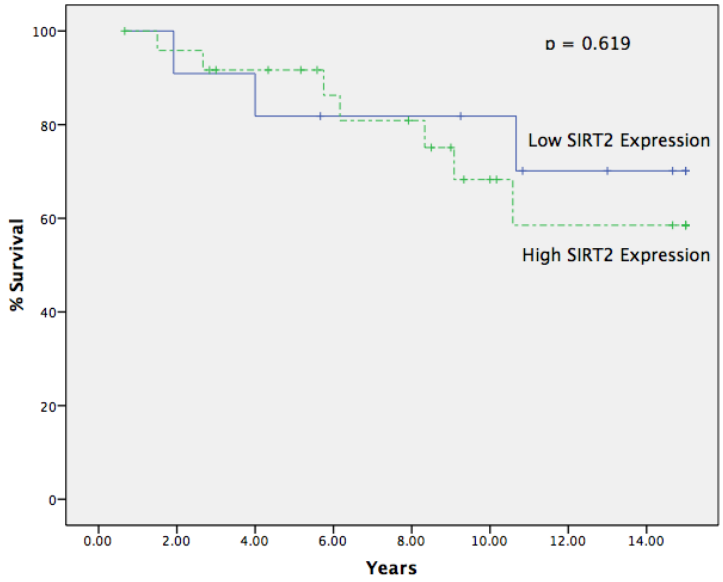

Figure 3.37: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT2 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows nonsignificant difference in the survival period in patients with tumours that express high levels of SIRT2 (n = 25, 11.72 years, 95% C.I. 9.75 - 15.07) when compared to those whose tumours express low levels of SIRT2 (n = 11, 12.30 years, 95% C.I. 9.53 – 15.07, Kaplan-Meier p = 0.619).

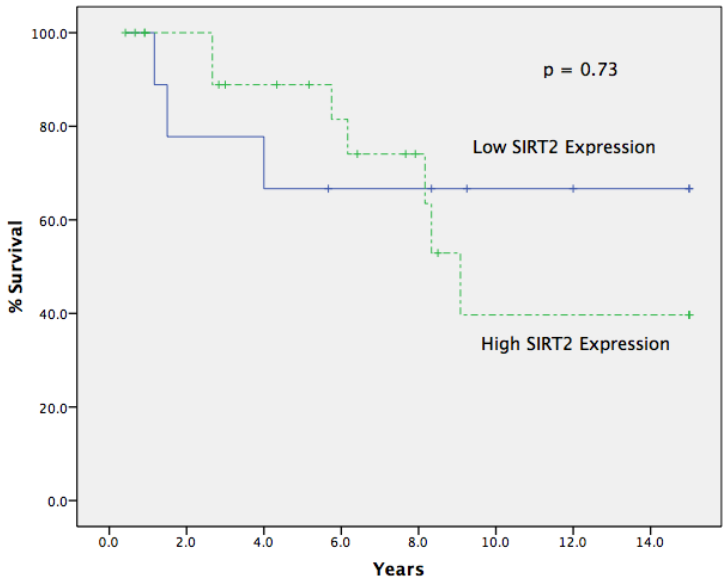

Figure 3.38: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT2 expression in patients with ER^{+ve} tumours.

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows no significant difference in the survival period in patients with tumours that express high levels of SIRT2 (n = 22, 10.74 years, 95% C.I. 6.78-14.7) when compared to those whose tumours express low levels of SIRT2 (n = 9, 10.07 years, 95% C.I. 7.58 – 12.57, Kaplan-Meier p = 0.73.

3.2.5.2 SIRT2 and recurrence.

3.2.5.2.1 Recurrence analysis in entire patient cohort according to SIRT2 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, in all breast cancer patients, patient numbers are shown in Table 3.9. There was no significant differences in the recurrence interval between patients with tumours that expressed high levels of SIRT2 (n = 48, 11.15 years, 95% C.I. 9.27-13.0) when compared to those whose tumours expressed low levels of SIRT*2* (n = 22, 10.95 years, 95% C.I. 8.22 -13.68, Kaplan-Meier p = 0.947, Figure 3.39).

Table 3.9: Number of breast cancer patient and tumour recurrences of all breast cancer patients groups included in the Kaplan-Meier survival analysis

SIRT2 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT2 gene expression	22	6	12	4	10	5
High SIRT2 gene expression	48	13	25	9	22	6
Overall	70	19	27	13	32	11

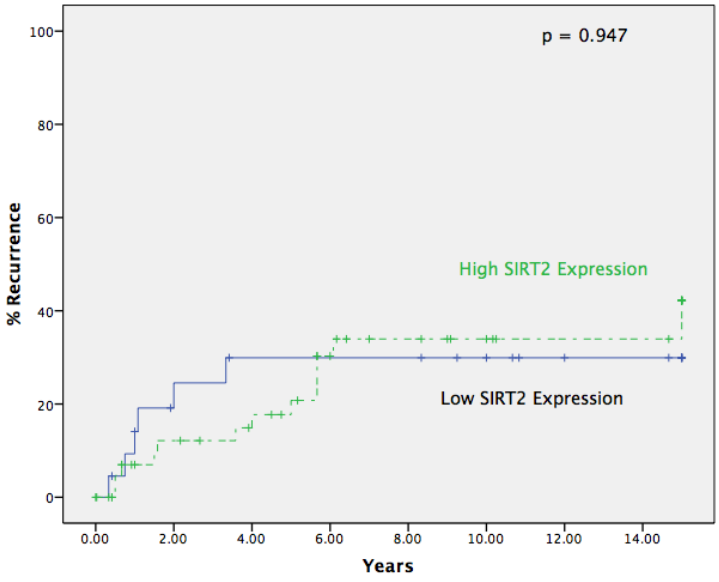

Figure 3.39: Kaplan-Meier recurrence plot for breast cancer patients according to the level of *SIRT2* expression.

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed a non significant decrease in the time to recurrence in patients with tumours that express low levels of SIRT2 (n = 22, 10.95 years, 95% C.I. 8.22 -13.68, when compared to those whose tumours express high levels of *SIRT2* (n = 48, 11.15 years, 95% C.I. 9.27-13.0, Kaplan-Meier p = 0.947).

3.2.5.2.2 Recurrence analysis in NPI selected patients according to SIRT2 levels.

When tumour recurrence among the NPI-selected patient group was analysed, there was also no significant difference according to the level of SIRT2 gene expression. Patient numbers are shown in Table 3.9 and the recurrence data in Figure (3.40). Time to recurrence was not significantly shorter in patients with tumours that expressed high levels of SIRT2 (n = 25, 10.51 years, 95% C.I. 7.87-13.16) when compared to those whose tumours express low levels of SIRT2 (n = 12, 10.56 years, 95% C.I. 6.99 -14.13, Kaplan-Meier p = 0.764, Figure 3. 40).

3.2.5.2.3 Recurrence analysis in ER $^{+ve}$ patients according to SIRT2 levels.

Further analysis of recurrence within the subgroup of patients with oestrogen receptor positive tumours (ER^{+ve}) failed to reveal any significant difference in time to recurrence between patients with tumours that expressed high levels of SIRT2 (n = 22, 10.46 years, 95% C.I. 7.58-13.35) when compared to those whose tumours express low levels of SIRT2 (n = 10, 8.24 years, 95% C.I. 4.02-12.45, Kaplan-Meier p = 0.352. Figure 3. 41). Patient numbers are shown in (Table 3.9).

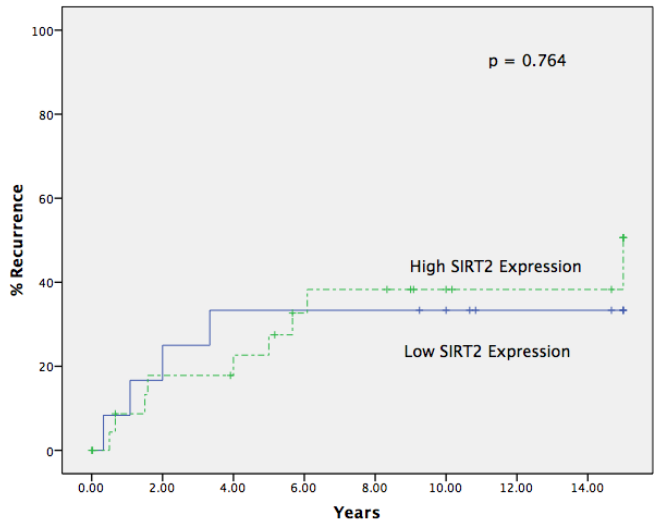

Figure 3.40: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT2 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed a insignificant decrease in the time to recurrence in patients with tumours that express high levels of SIRT2 (n = 25, 10.51 years, 95% C.I. 7.87-13.16) when compared to those whose tumours express low levels of SIRT2 (n = 12, 10.56 years, 95% C.I. 6.99 -14.13, Kaplan-Meier p = 0.764.

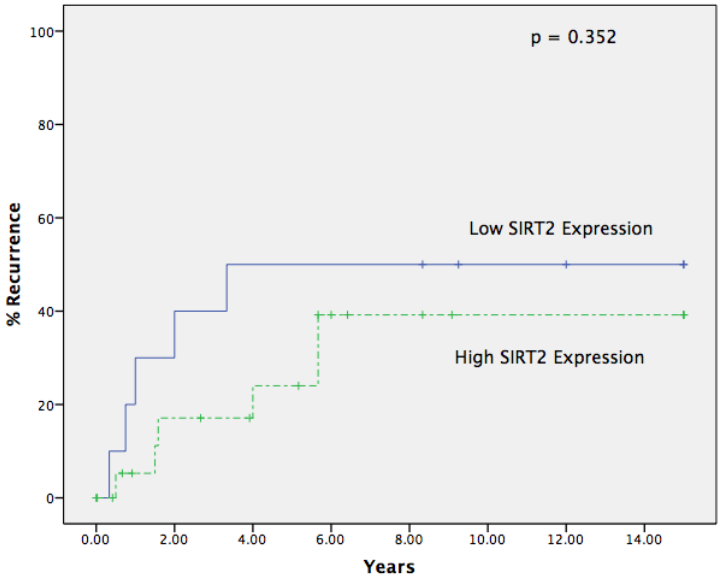

Figure 3.41: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT2 expression in patients with ER^{+ve} tumours.

High and low SIRT2 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed a non significant decrease in the time to recurrence in patients with tumours that express high levels of SIRT2 (n = 22, 10.46 years, 95% C.I. 7.58-13.35) when compared to those whose tumours express low levels of SIRT2 (n = 10, 8.24 years, 95% C.I. 4.02-12.45, Kaplan-Meier p = 0.352).

3.2.6 Conclusions.

This study investigated the relative transcriptional expression levels of the SIRT2 gene in (24) normal, (75) non-malignant and (73) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT2 as the data distributions were positively skewed in all patient groups.

The data for SIRT2 gene expression variation between breast biopsies showed that there were significant differences in SIRT2 gene expression between biopsies from malignant, non-malignant and normal patient groups in a three-way comparison. The expression level of SIRT2 is significantly lower in malignant compared to both non-malignant and normal patient groups. SIRT2 gene expression does not differ significantly between the non-malignant and normal patient groups, which differ in their age distributions (Table 3.6).

There was a significant correlation between decreased SIRT2 gene expression levels and higher tumour grade.

SIRT2 gene expression did not show significant correlation with patient survival and tumour recurrence, either in the entire patient set, or in a patient group selected by NPI ,or ER^{+ve} status, to have an intermediate clinical prognosis.

3.3 The relative transcriptional expression levels of SIRT3 in breast biopsies.

3.3.1 The relative transcriptional expression levels of SIRT3 and patient's age.

The relative transcriptional expression levels of the SIRT3 gene were determined in 25 normal biopsies, 75 non-malignant and 72 malignant patient biopsies (Table 3.10).

Table 3.10 Patient biopsies used in the SIRT3 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT3 Expression	Normal	25	96.20%	1	3.80%	26	100.00%
	Non-Malignant	75	96.20%	3	3.80%	78	100.00%
	Malignant	72	98.60%	1	1.40%	73	100.00%

The correlation between the level of SIRT3 relative transcriptional expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3. 42a). The median age of this patient group was 51 years (20years - 84years). This data showed that there was no significant association between the relative transcription levels of SIRT3 and chronological age (Spearman's rank correlation coefficient; p = 0.766; Figure 3. 42b).

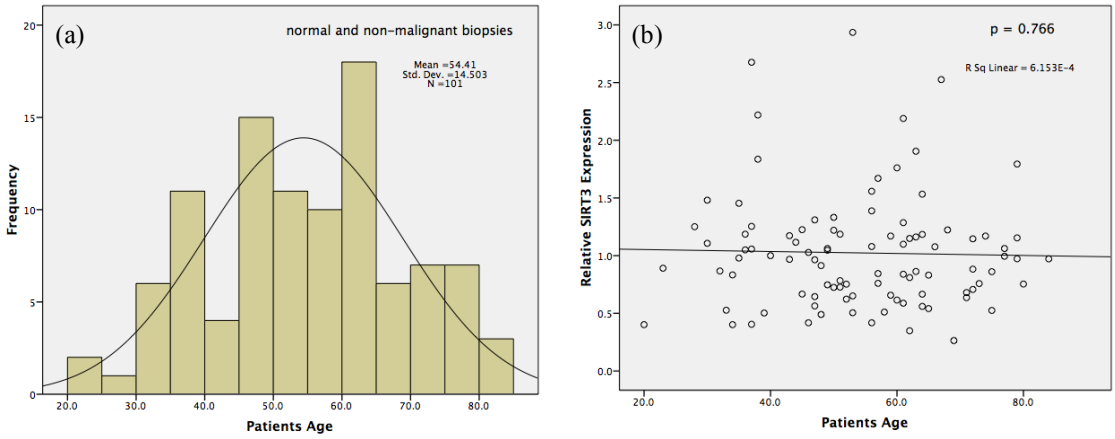

Figure 3.42: (a) Distribution of patient's age in patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT3 and patient's age in patient group that combines both normal and non-malignant patients (Spearman test, p = 0.766).

3.3.2 Data distribution of the relative transcriptional expression levels of SIRT3.

The distribution of the relative transcriptional gene expression of SIRT3 was investigated in the three different studied biopsy types (Figure 3. 43). The histograms of the relative transcriptional gene expression of SIRT3 distribution showed that the range of the SIRT3 gene expression values was not normally distributed; instead the SIRT3 gene expression distribution was positive skewed in all groups (Figure 3. 43). Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT3.

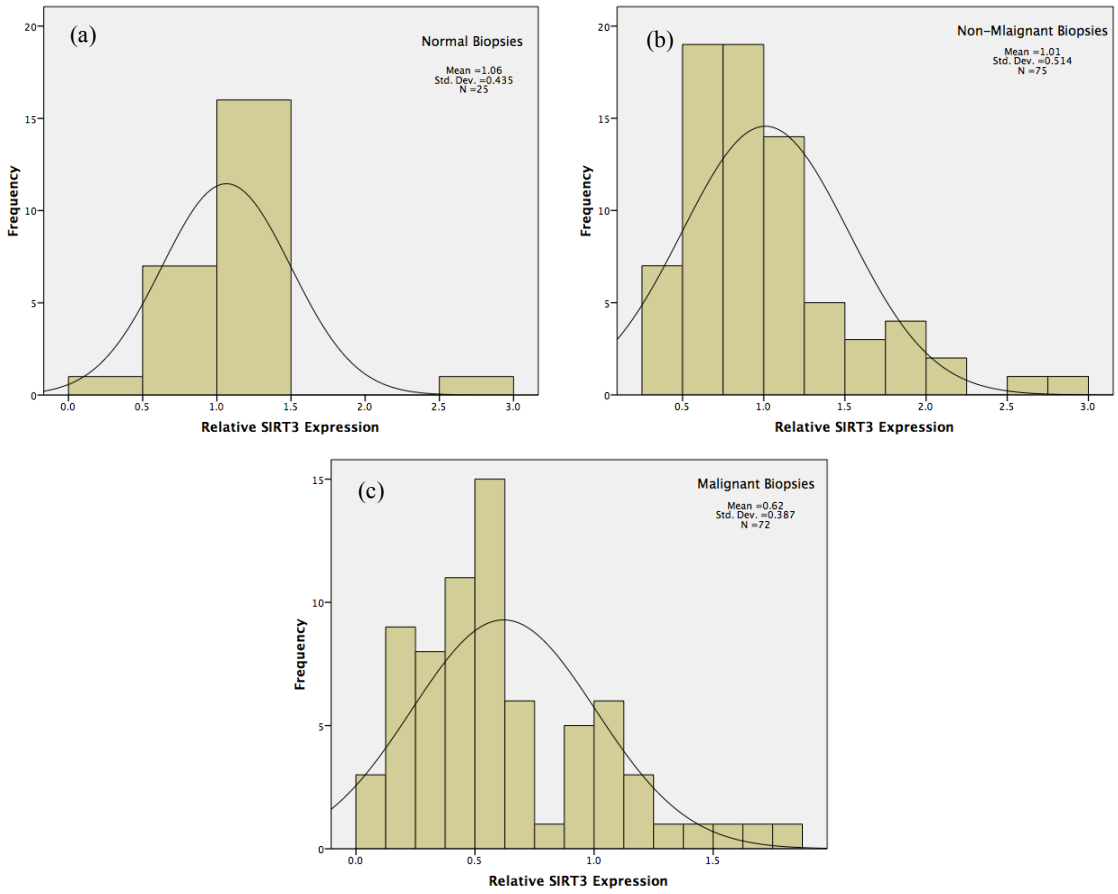

Figure 3.43: Distribution of the relative transcriptional gene expression of SIRT3 in three different groups.

(a) Relative transcriptional gene expression of SIRT3 in normal breast biopsies, (b) Relative transcriptional gene expression of SIRT3 in non-malignant breast biopsies, (c) Relative transcriptional gene expression of SIRT3 in malignant breast biopsies.

3.3.3 Comparison of the relative transcriptional expression levels of SIRT3 between patient groups.

The relative transcriptional expression levels of SIRT3 were compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT3 was significantly different between the three groups of breast biopsies ($p < 0.001$, Figure 3. 44). This experiment showed that malignant samples have significantly lower levels of SIRT3 gene expression compared to both non-malignant and normal samples ($p < 0.001$, Figure 3. 44).

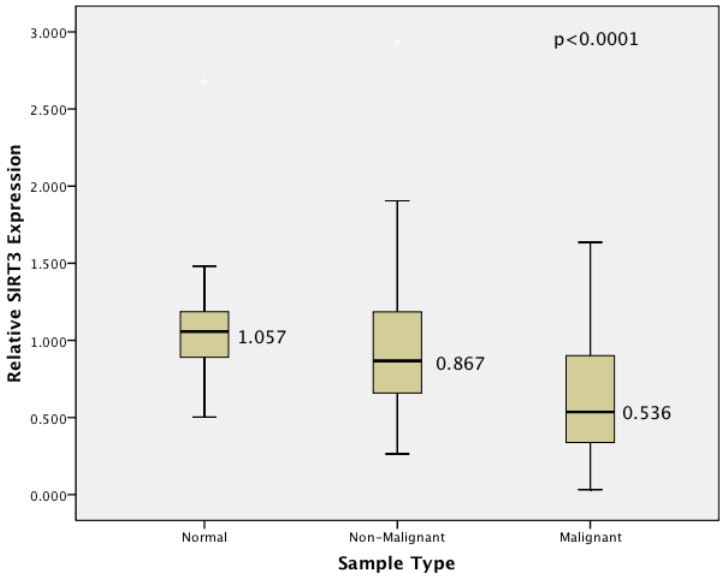

Figure 3.44: Box plot showing the transcriptional expression of SIRT3 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

The median value for each patient group is shown to the right of each box plot ($p < 0.001$).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6) showed that the malignant group differed significantly in SIRT3 gene expression from both non-malignant and normal groups (malignant versus non-malignant $p < 0.001$, malignant versus normal; $p < 0.001$). However, there was no significant difference between the normal and non-malignant groups ($p = 0.301$).

3.3.4 Comparison of the relative transcriptional expression levels of SIRT3 according to histopathological markers

The relationships between SIRT3 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.3.4.1 Comparison of the relative transcriptional expression levels of SIRT3 according to tumour grades.

Analysis of the data for the relationship between SIRT3 gene expression and tumour grade showed a significant difference in the relative transcriptional expression levels of SIRT3 between all three tumour grades when compared together (Kruskal-Wallis test $p < 0.049$) (Figure 3. 45).

Pairwise comparisons of the three groups using the Mann-Whitney test showed that grade 3 tumours groups differed significantly in SIRT3 gene expression from grade 2 ($p = 0.022$) but not from grade 1 tumours ($p = 0.217$, Table 3.6). This could be attributed to the small number of patients (3) included in grade 1 groups. However, it could be said that the overall trend toward decreased levels of SIRT3 expression with increasing tumour grade is consistent. Thus the expression of SIRT3 decreases with increasing severity (higher grade) of tumour. There was no significant difference between grade 2 and grade 1 tumours ($p = 0.717$; Figure 3. 45).

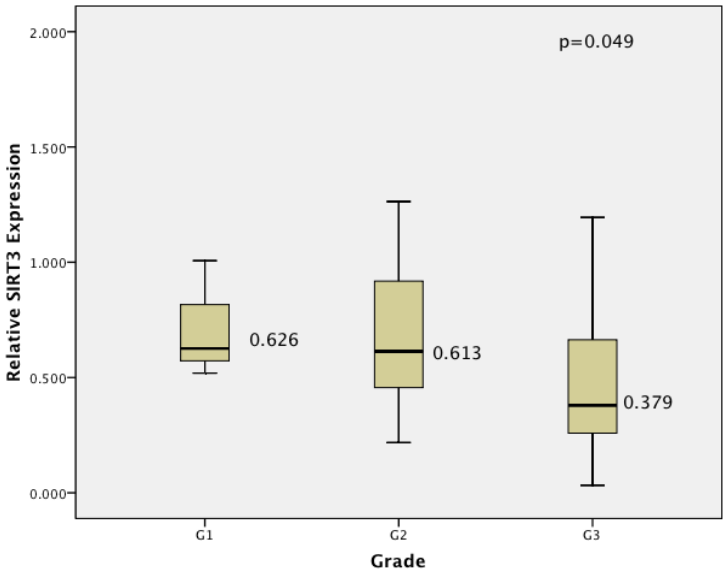

Figure 3.45: Box plot showing the relative transcriptional expression of in breast cancer biopsies by tumour grade.

n = 3 for grade 1, n = 32 for grade 2 and n = 31 for grade 3 *(*Kruskal Wallis Test, *p = 0.001)*. The median value for each patient group is shown to the right of each box plot.

3.3.4.2 Comparison of SIRT3 gene transcription levels according to tumour size, nodal status, oestrogen receptor status and lymphovascular invasion.

Analysis of the data for the relationship between SIRT3 gene expression and tumour size, nodal status, oestrogen receptor status and lymphovascular invasion all showed no significant differences in the relative transcriptional expression levels of SIRT3 between any categories when compared together (Figure 3. 46 a, b, c, and d). Table 3.6 shows the Kruskal-Wallis test p values for all comparisons.

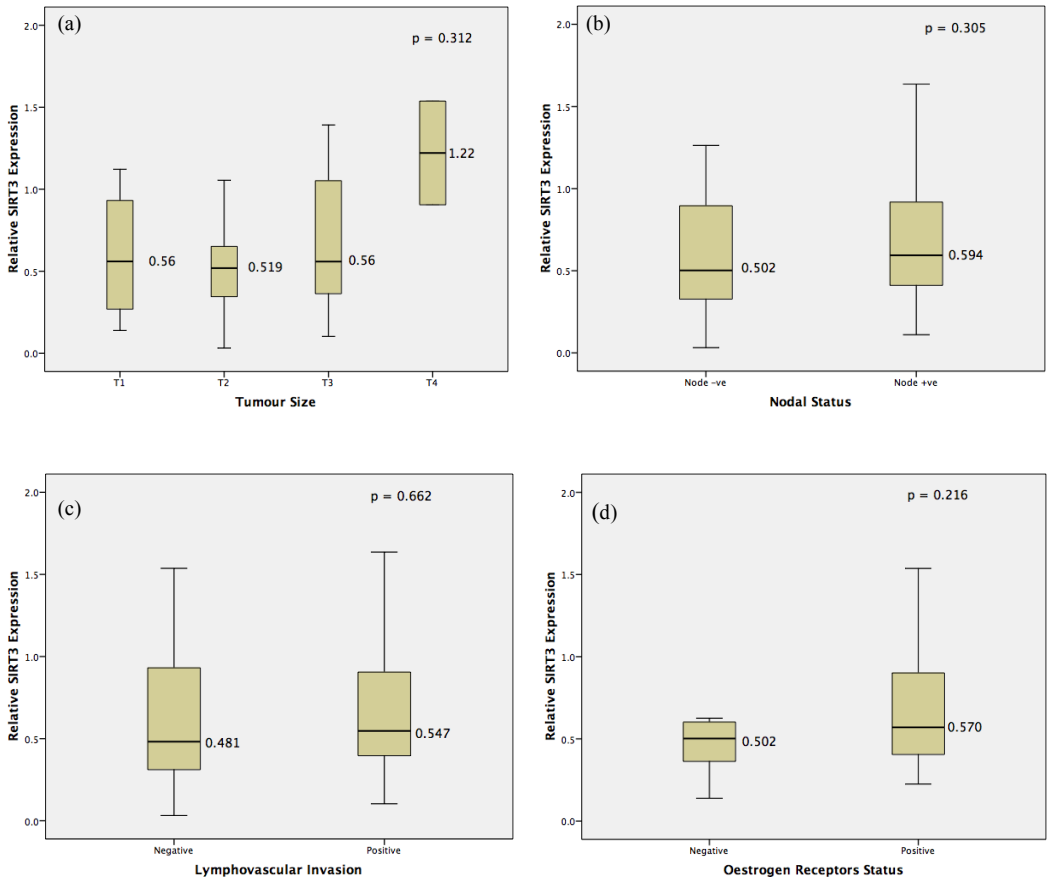

Figure 3.46: Box plots showing the relative transcriptional expression of SIRT3 in breast cancer biopsies by:

(a) tumour size, n = 10 for T1, n = 45 for T2 , n = 14 for T3 and 2 for T4 *(* Kruskal Wallis Test, p = 0.312*),* (b) Nodal status, n = 21 for node –ve and n = 44 for node +ve (Mann Whitney test p = 0.305) , (c) Oestrogen receptor status, n = 21 for ER^{-ve} and n = 32 for ER^{+ve} (Mann Whitney test p = 0.216). (d) Lymphovascular invasion (LV) n = 18 for LV^{-ve} and n = 29 for LV^{+ve} (Mann Whitney test p = 0.662). The median value for each patient group is shown right of each box.

3.3.5 The relationship of the relative transcriptional expression levels of SIRT3 to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT3 relative gene expression, the patients were grouped in tertiles according to the level of SIRT3 gene expression. In analyses shown below, the two tertiles with lower SIRT3 gene expression levels have been combined and are compared to the highest levels of SIRT3 gene expression tertile group. Table 3.11 and Table 3.12 show the numbers of patients included in each group.

3.3.5.1 SIRT3 and survival

3.3.5.1.1 Survival analysis in entire patient cohort according to SIRT3 levels.

Analysis of the survival data for the entire patient group using the Kaplan-Meier test shows no significant difference in survival period when all patients are included (Table 3.11). The survival period was non-significantly shorter in patients with tumours that expressed high levels of SIRT3 (n = 24, 10.75 years, 95% C.I. 8.2 -12.76) when compared to those whose tumours expressed low levels of SIRT3 (n = 45, 11.13 years, 95% C.I. 9.5– 12.76, Kaplan-Meier p = 0.789; Figure 3. 47).

Table 3.11: Number of breast cancer patient and cancer specific deaths of all breast cancer patient groups included in the Kaplan-Meier survival analysis.

SIRT3 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT3 gene expression	45	14	24	7	17	7
High SIRT3 gene expression	24	7	12	3	13	3
Overall	69	21	36	10	30	10

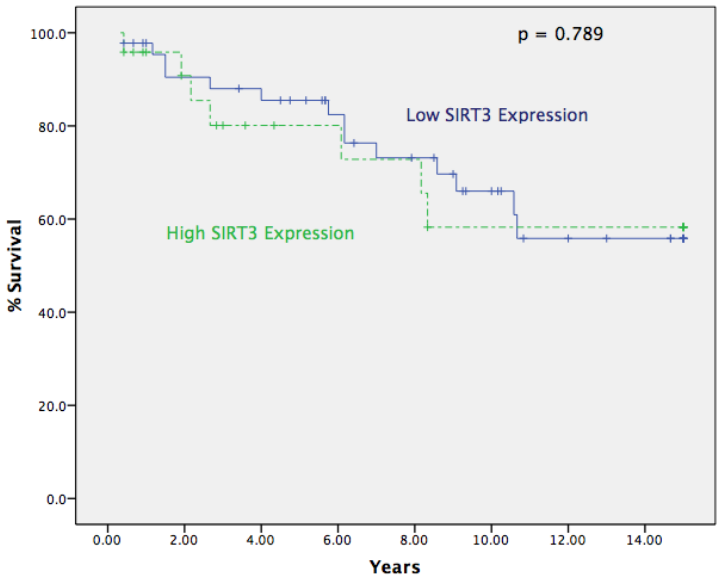

Figure 3.47: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT3 expression.

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a non significant decrease in the survival period for patients with tumours that express high levels SIRT3 (n = 24, 10.75 years, 95% C.I. 8.2 - 12.76) when compared to those whose tumours express low levels of SIRT3 (n = 45, 11.13 years, 95% C.I. 9.5– 12.76, Kaplan-Meier p = 0.789).

3.3.5.1.2 Survival analysis in NPI selected patients according to SIRT3 levels.

Analysis of the survival data for the NPI-selected patient group (Table 3. 11) using the Kaplan-Meier test did not show any significant difference in the survival period in patients with tumours that expressed low levels of SIRT3 (n = 24, 12.02 years, 95% C.I. 10.19 - 13.84) when compared to those whose tumours expressed high levels of SIRT3 (n = 12, 11.78 years, 95% C.I. 8.67 – 14.88, Kaplan-Meier p = 0.968, Figure 3. 48).

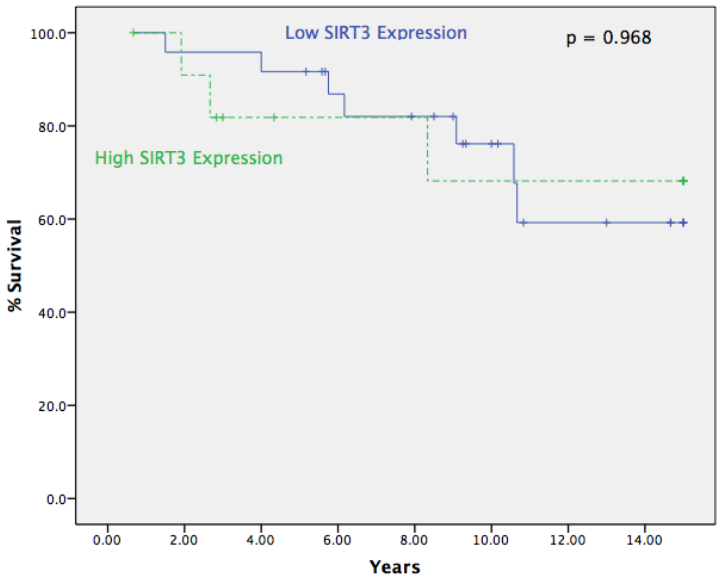

Figure 3.48: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT3 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows insignificant difference in the survival period in patients with tumours that express high levels of SIRT3 (n = 12, 11.78 years, 95% C.I. 8.67 – 14.88), when compared to those whose tumours express low levels of SIRT3 (n = 24, 12.02 years, 95% C.I. 10.19 -13.84; Kaplan-Meier p = 0.968).

3.3.5.1.3 Survival analysis in ER^{+ve} selected patients according to SIRT3 levels.

Analysis of the survival data for the ER^{+ve} patient group (Table 3. 11) using the Kaplan-Meier test showed no significant difference in the survival period in patients with tumours that expressed low levels of SIRT3 (n = 17, 9.76 years, 95% C.I. 6.91-12.62) when compared to those whose tumours expressed high levels of SIRT3 (n = 13, 11.74 years, 95% C.I. 8.78 – 14.70, Kaplan-Meier p = 0.487, Figure 3.46).

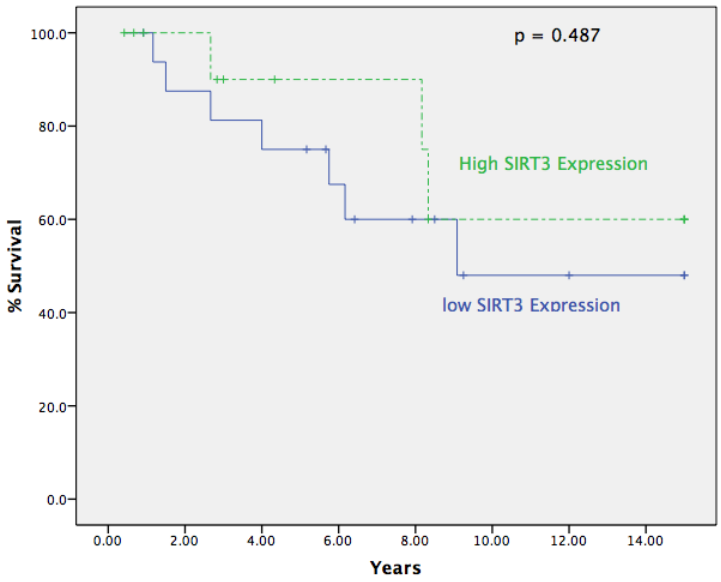

Figure 3.49: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT3 expression in patients with ER^{+ve} tumours.

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows no significant difference in the survival period in patients with tumours that express low levels of SIRT3 (n = 17, 9.76 years, 95% C.I. 6.91-12.62) when compared to those whose tumours express high levels of SIRT3 (n = 13, 11.74 years, 95% C.I. 8.78 – 14.70, Kaplan-Meier p = 0.487).

3.3.5.2 SIRT3 and Recurrence.

3.3.5.2.1 Recurrence analysis in entire patient cohort according to SIRT3 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, in all breast cancer patients, patient numbers are shown in Table 3.12. There was no significant differences in the recurrence interval between patients with tumours that expressed high levels of SIRT3 (n = 23, 10.91 years, 95% C.I. 7.67-14.15) when compared to those whose tumours expressed low levels of SIRT3 (n = 46, 11.03 years, 95% C.I. 9.23 -12.8, Kaplan-Meier p = 0.866, Figure 3. 50)

Table 3.12: Number of breast cancer patient and tumour recurrences of all breast cancer patient groups included in the Kaplan-Meier survival analysis

SIRT3 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT3 gene expression	46	13	25	9	18	8
High SIRT3 gene expression	23	6	12	4	13	3
Overall	69	19	37	13	32	11

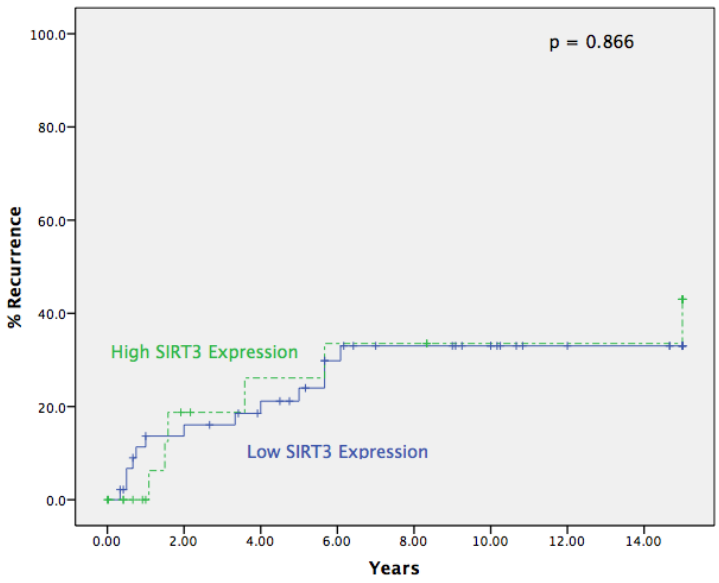

Figure 3.50: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT3 expression.

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a non significant difference in the time to recurrence in patients with tumours that express high levels of SIRT3 (n = 23, 10.91 years, 95% C.I. 7.67-14.15) when compared to those whose tumours express low levels of SIRT3 (n = 46, 11.03 years, 95% C.I. 9.23 -12.8, Kaplan-Meier p = 0.866.

3.3.5.2.2 Recurrence analysis in NPI selected patients according to SIRT3 levels.

When tumour recurrence among the NPI-selected patient group was analysed (Table 3. 12), there was also no significant difference according to the level of SIRT3 gene expression in patients with tumours that expressed low levels of SIRT3 (n = 25, 10.51 years, 95% C.I. 8.19-12.89) when compared to those whose tumours expressed high levels of SIRT3 (n = 12, 10.46 years, 95% C.I. 5.62 -15.30, Kaplan-Meier p = 0.903, Figure 3.51).

3.3.5.2.3 Recurrence analysis in ER^{+ve} patients according to SIRT3 levels.

Further analysis of recurrence within the subgroup of patients with ER^{+ve} tumours (Table 3.14) failed to reveal any significant difference in time to recurrence between patients with tumours that expressed low levels of SIRT3 (n = 18, 8.96 years, 95% C.I. 5.86 -12.05) when compared to those whose tumours express high levels of SIRT3 (n = 13, 10.46 years, 95% C.I. 6.33-14.6, Kaplan-Meier p = 0.519, Figure 3. 52).

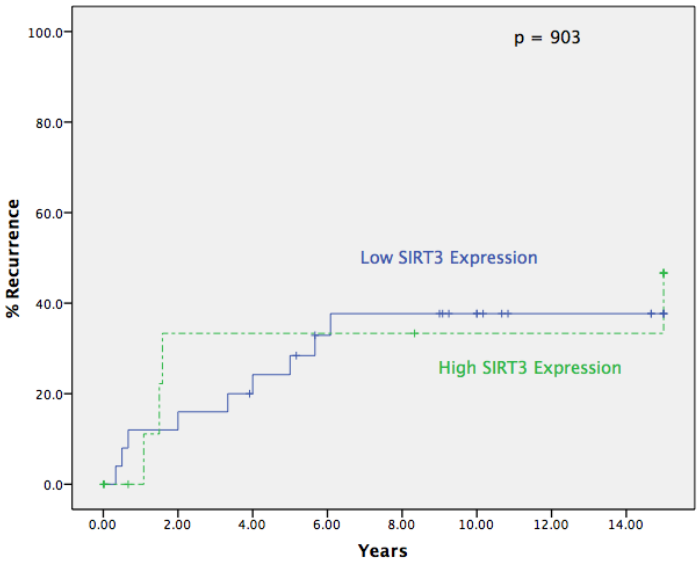

Figure 3.51: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT3 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a non significant difference in the time to recurrence period in patients with tumours that express low levels of SIRT3 (n = 25, 10.51 years, 95% C.I. 8.19-12.89) when compared to those whose tumours express high levels of SIRT3 (n = 12, 10.46 years, 95% C.I. 5.62 -15.30, Kaplan-Meier p = 0.903).

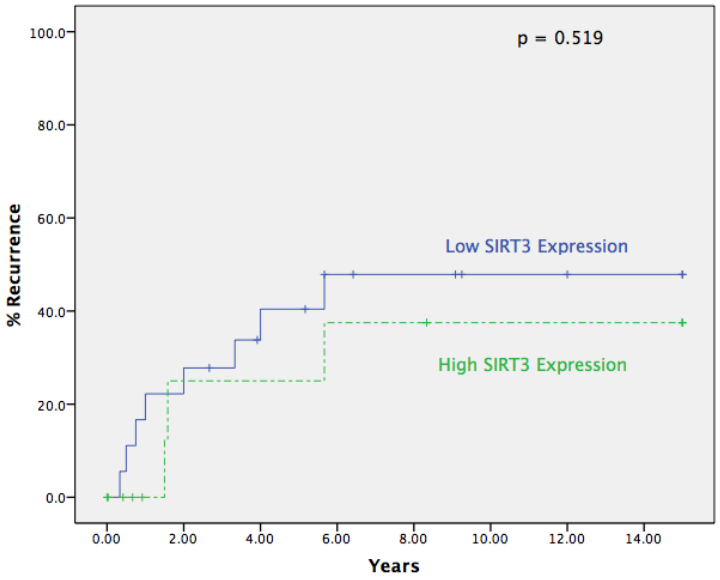

Figure 3.52: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT3 expression in patients with ER^{+ve} tumours.

High and low SIRT3 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a insignificant difference in the time to recurrence in patients with tumours that express low levels of SIRT3 (n = 18, 8.96 years, 95% C.I. 5.86 -12.05) when compared to those whose tumours express high levels of SIRT3 (n = 13, 10.46 years, 95% C.I. 6.33-14.6, Kaplan-Meier p = 0.519).

3.3.6 Conclusion.

This study investigated the relative transcriptional expression levels of the SIRT3 gene in (25) normal, (75) non-malignant and (72) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT3 as the data distributions were positively skewed in all patient groups.

The data for SIRT3 gene expression variation between breast biopsies showed that there were significant differences in SIRT3 gene expression between biopsies from malignant, non-malignant and normal patient groups in a three-way comparison. The expression level of SIRT3 is significantly lower in malignant compared to both non-malignant and normal patient groups. SIRT3 gene expression does not differ significantly between the non-malignant and normal patient groups (Table 3.6), which differ in their age distributions.

There was a significant correlation between decreased SIRT3 gene expression level and higher tumour grade.

SIRT3 gene expression did not show significant correlation with patient survival or tumour recurrence either in the entire patient set or in a patient group selected by NPI, or ER^{+ve} status, to have an intermediate clinical prognosis.

3.4 The relative transcriptional expression levels of SIRT4 in breast biopsies.

3.4.1 The relative transcriptional expression levels of SIRT4 and patients age.

The relative transcriptional expression levels of the SIRT4 gene were determined in 25 normal biopsies, 72 non-malignant and 69 malignant patient biopsies (Table 3.13).

Table 3.13: Patient biopsies used in the SIRT4 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT4 Expression	Normal	25	96.20%	1	3.80%	26	100.00%
	Non-Malignant	72	92.30%	6	7.70%	78	100.00%
	Malignant	69	94.50%	4	5.50%	73	100.00%

The correlation between the level of SIRT4 relative transcriptional expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3.53 a). The median age of this patient group was 51years (20 years – 84 years). This data showed that there was a significant association between increasing levels of SIRT4 relative transcription and chronological age (Spearman's rank correlation coefficient; $p < 0.001$; Figure 3. 53b).

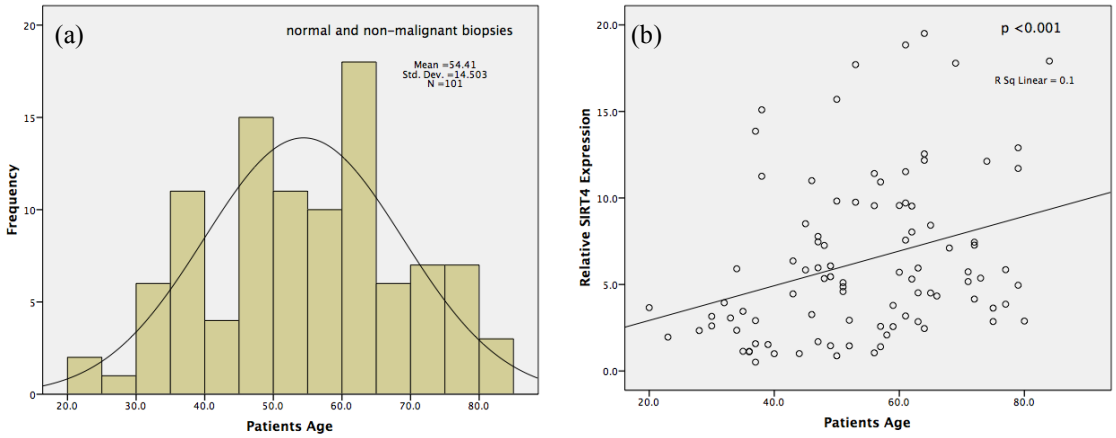

Figure 3.53: (a) Histogram shows the distribution of patient's age patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT4 and patient's age in patient group that combines both normal and non-malignant patients (spearman test, p <0.001).

3.4.2 Data distribution of the relative transcriptional expression levels of SIRT4.

The distribution of the relative transcriptional gene expression of SIRT4 was investigated in the three different studied biopsy types, (Figure 3. 54). The histograms of the relative transcriptional gene expression of SIRT4 distribution showed that the range of the SIRT4 gene expression values was not normally distributed; instead the SIRT4 gene expression distribution was positive skewed in all groups (Figure 3. 54 a, b and c). Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT4.

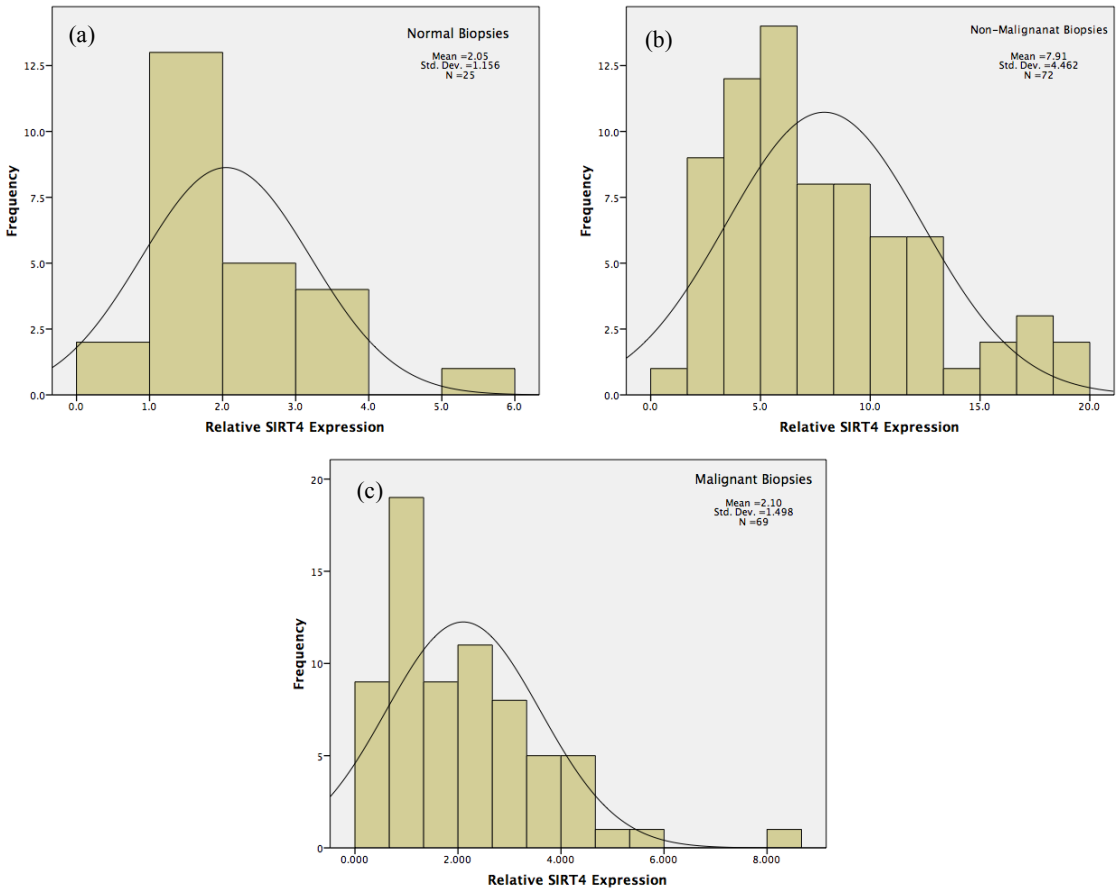

Figure 3.54: Distribution of the relative transcriptional gene expression of SIRT4 in three different groups.

(a) Relative transcriptional gene expression of SIRT4 in normal breast biopsies. (b) Relative transcriptional gene expression of SIRT4 in non-malignant breast biopsies. (c) Relative transcriptional gene expression of SIRT4 in malignant breast biopsies.

3.4.3 Comparison of the relative transcriptional expression levels of SIRT4 between patient groups.

The level of SIRT4 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT4 was significantly different between the three groups of breast biopsies ($p < 0.001$, (Figure 3. 55). This experiment showed that non-malignant samples have significantly higher levels of SIRT4 gene expression compared to both malignant and normal samples ($p < 0.001$, Figure 3. 55).

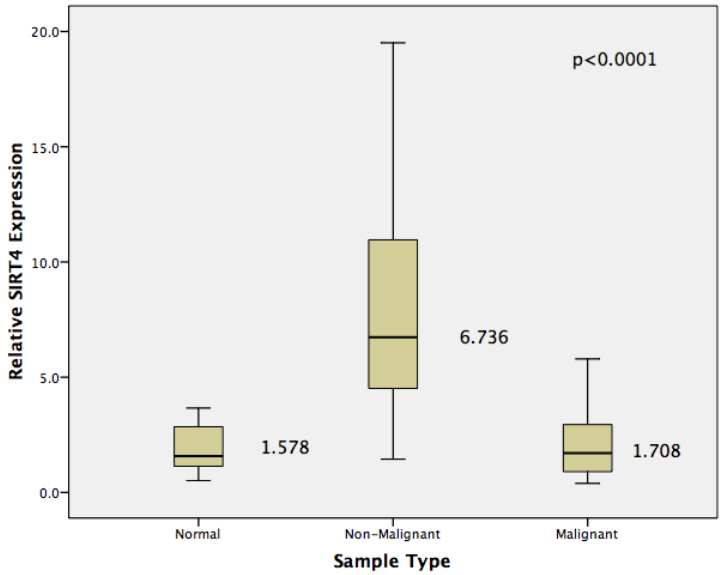

Figure 3.55: Box plot showing the transcriptional expression of SIRT4 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

The median value for each patient group is shown to the right of each box plot ($p < 0.001$).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6) showed that the non-malignant group differed significantly in SIRT4 gene expression from both malignant and normal groups (non-malignant versus malignant $p < 0.001$, non-malignant versus normal; $p < 0.001$). However, there was no significant difference between the malignant and normal groups ($p = 0.729$).

3.4.4 Comparison of SIRT4 gene transcription levels according to histopathological markers.

The relationships between SIRT4 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.4.4.1 Comparison of SIRT4 gene transcription levels according to nodal status.

Analysis of the data for the relationship between SIRT4 gene expression and nodal status showed a marginally significant difference in the relative transcriptional expression levels of SIRT4 between node positive and node negative tumours (Mann- Whitney $p < 0.059$; Figure 3. 56).

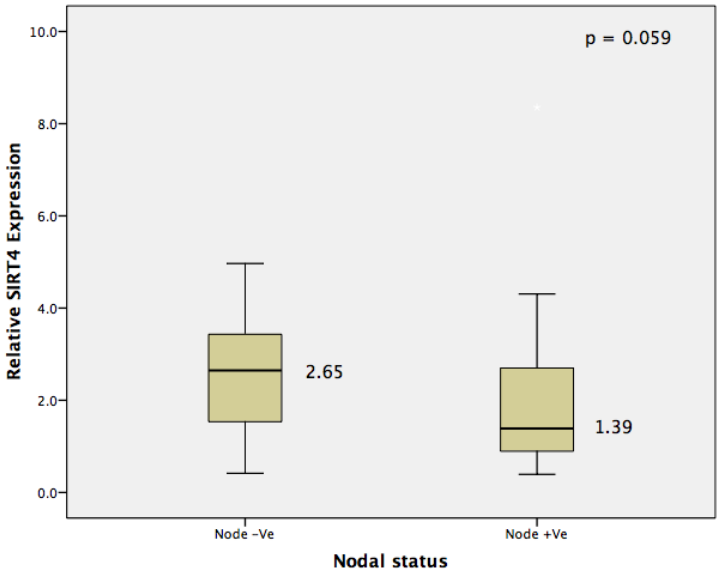

Figure 3.56: Box plot showing the relative transcriptional expression of in breast cancer biopsies by nodal status.

n = 41 for node positive tumours, n = 19 for node negative tumours *(*Mann- Whitney test, *p = 0.059)*. The median value for each patient group is shown to the right of each box plot.

3.4.4.2 Comparison of the relative transcriptional expression levels of SIRT4 according to tumour grade, tumour size, oestrogen receptor status and lymphovascular invasion

Analysis of the data for the relationship between SIRT4 gene expression and tumour grade, tumour size, ER status and lymphovascular invasion all showed no significant differences in the relative transcriptional expression levels of SIRT4 between any categories when compared together (Figure 3. 57 a, b, c and d). Table 3.6 shows the Kruskal-Wallis test p values for all comparisons.

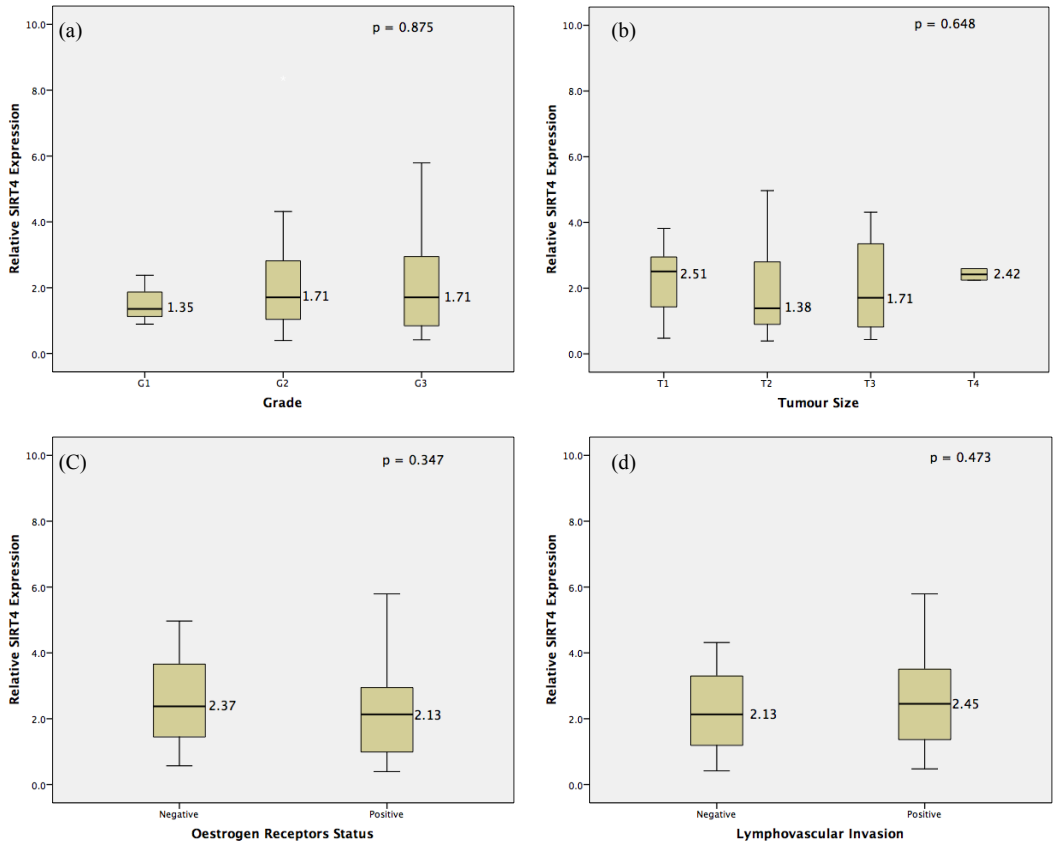

Figure 3.57: Box plots showing the relative transcriptional expression of SIRT4 in breast cancer biopsies by known prognostic markers:

(a) tumour grade, n = 3 for G1, n = 32 for G2, and n = 29 for G3 (Mann Whitney test p = 0.875). (b) tumour size, n = 10 for T1, n = 43 for T2 , n = 13 for T3 and n = 2 for T4 *(*Kruskal Wallis Test, p = 648*)*, (c) oestrogen receptor status, n = 20 for ER^{-ve} and n = 32 for ER^{+ve}(Mann Whitney test p = 0.347. (d) Lymphovascular invasion (LV) n = 18 for LV^{-ve} and n = 27 for LV^{+ve} (Mann Whitney test p = 0.473). The median value for each patient group is shown right of each box.

3.4.5 The relationship of the relative transcriptional expression levels of SIRT4 in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT4 relative gene expression, the patients were grouped in tertiles according to the level of SIRT4 gene expression. In the analysis shown below, the two tertiles with higher levels of SIRT4 gene expression have been combined and are compared to the lowest level of SIRT4 gene expression tertile group. Table 3.14 and Table 3.15 show the numbers of patients included in each group.

3.4.5.1 SIRT4 and survival.

3.4.5.1.1 Survival analysis in entire patient cohort according to SIRT4 levels.

Analysis of the survival data for the entire patient group using the Kaplan-Meier test shows no significant difference in survival period when all patients are included (Table 3. 14). The survival period was non-significantly shorter in patients with tumours that expressed low levels of SIRT4 (n = 22, 10.07 years, 95% C.I. 7.42 -12.72) when compared to those whose tumours expressed low levels of SIRT4 (n = 44, 11.65 years, 95% C.I. 10.09 – 13.21, Kaplan-Meier p = 0.38, Figure 3. 58).

Table 3.14: Number of breast cancer patient and cancer specific deaths of all breast cancer patient groups included in the Kaplan-Meier survival analysis.

SIRT4 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT4 gene expression	22	8	10	4	8	4
High SIRT4 gene expression	44	12	25	5	22	6
Overall	66	20	35	9	30	10

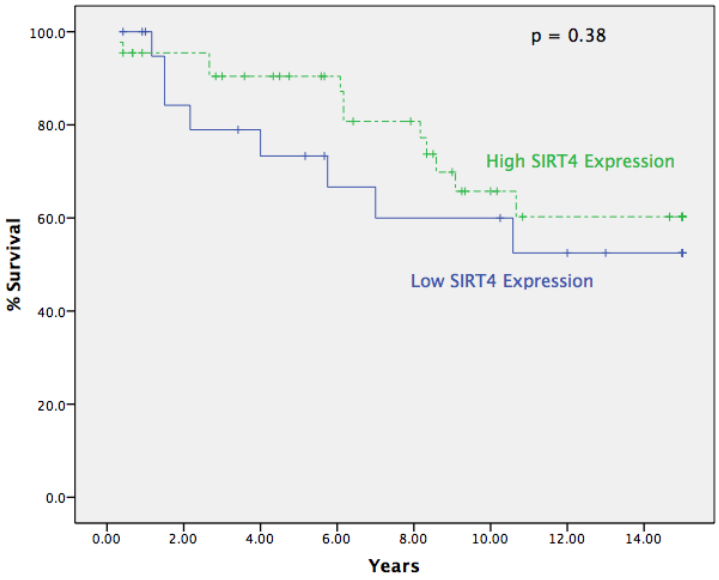

Figure 3.58: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT4 expression.

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows a non significant decrease in the survival period for patients with tumours that express low levels of SIRT4 (n = 22, 10.07 years, 95% C.I. 7.42 - 12.72) when compared to those whose tumours express low levels of SIRT4 (n = 44, 11.65 years, 95% C.I. 10.09 – 13.21, Kaplan-Meier p = 0.38).

3.4.5.1.2 Survival analysis in NPI selected patients according to SIRT4 levels.

Analysis of the survival data for the NPI-selected patient group (Table 3.14) using the Kaplan-Meier test did not show any significant difference in the survival period in patients with tumours that express low levels of SIRT4 (n = 10, 10.99 years, 95% C.I. 7.73 -14.23) when compared to those whose tumours express high levels of SIRT4 (n = 25, 12.82 years, 95% C.I. 11.18 – 14.47, Kaplan-Meier p = 0.384; Figure 3. 59).

3.4.5.1.3 Survival analysis in ER^{+ve} selected patients according to SIRT4 levels.

Analysis of the survival data for the ER^{+ve} patient group (Table 3.14) using the Kaplan-Meier test showed non-significant difference in the survival period in patients with tumours that expressed low levels of SIRT4 (n = 8, 7.76 years, 95% C.I. 3.18 -12.34) when compared to those whose tumours expressed high levels of SIRT4 (n = 22, 11.32 years, 95% C.I. 9.02 – 13.62, Kaplan-Meier p = 0.135; Figure 3. 60).

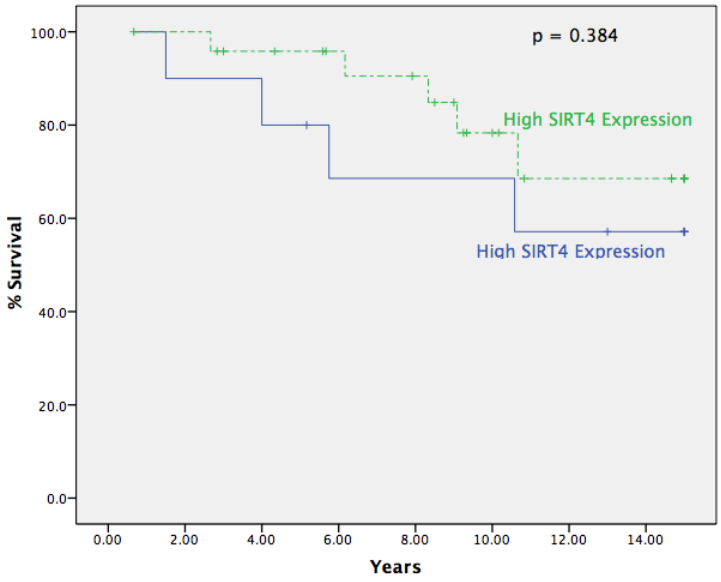

Figure 3.59: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT4 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows insignificant difference in the survival period in patients with tumours that express low levels of SIRT4 (n = 10, 10.99 years, 95% C.I. 7.73 - 14.23) when compared to those whose tumours express high levels of SIRT4 (n = 25, 12.82 years, 95% C.I. 11.18 – 14.47, Kaplan-Meier p = 0.384).

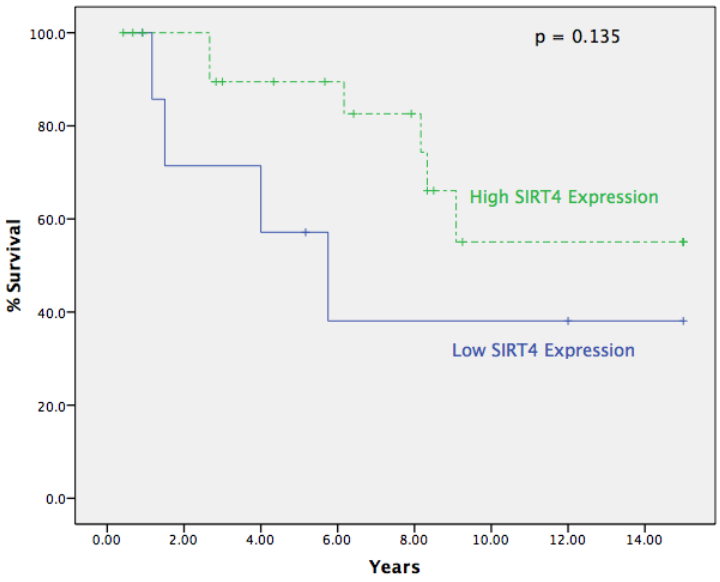

Figure 3.60: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT4 expression in patients with ER^{+ve} tumours.

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The survival curve shows no significant difference in the survival period in patients with tumours that express low levels of SIRT4 (n = 8, 7.76 years, 95% C.I. 3.18 - 12.34) when compared to those whose tumours express high levels of SIRT4 (n = 22, 11.32 years, 95% C.I. 9.02 – 13.62, Kaplan-Meier p = 0.135).

3.4.5.2 SIRT4 and Recurrence.

3.4.5.2.1 Recurrence analysis in entire patient cohort according to SIRT4 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, in all breast cancer patients, patient numbers are shown in Table 3.15. Kaplan-Meier analysis showed that the time to recurrence was not significantly shorter in patients with tumours that expressed low levels of SIRT4 (n = 23, 9.89 years, 95% C.I. 6.89 -12.88, when compared to those whose tumours expressed high levels of SIRT4 (n = 43, 11.96 years, 95% C.I. 10.26-13.67), Kaplan-Meier p = 0.098; Figure 3. 61).

Table 3.15: Number of breast cancer patient and tumour recurrences of all breast cancer patient groups included in the Kaplan-Meier survival analysis

SIRT4 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT4 gene expression	23	9	11	6	9	6
High SIRT4 gene expression	43	9	25	6	22	5
Overall	66	18	36	12	31	11

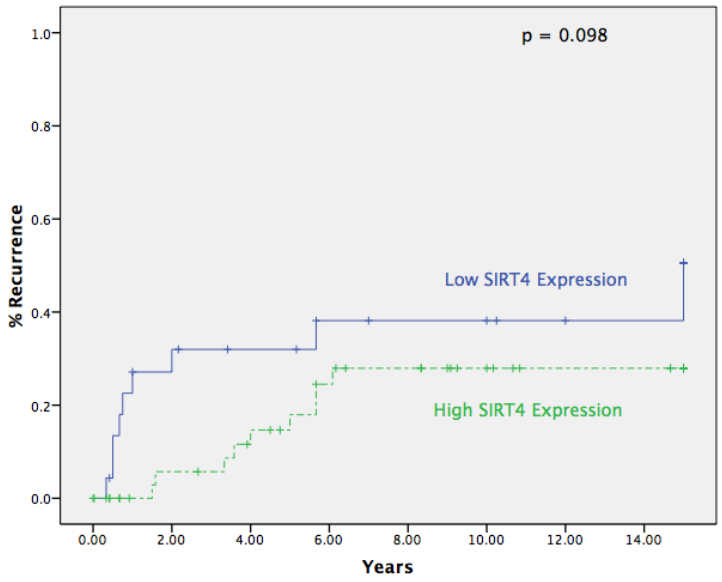

Figure 3.61: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT4 expression.

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed insignificant decrease in the time to recurrence in patients with tumours that express low levels of SIRT4 (n = 23, 9.89 years, 95% C.I. 6.89 - 12.88, when compared to those whose tumours express high levels of SIRT4 (n = 43, 11.96 years, 95% C.I. 10.26-13.67, Kaplan-Meier p = 0.098).

3.4.5.2.2 Recurrence analysis in NPI selected patients according to SIRT4 levels.

When tumour recurrence among the NPI-selected patient group was analysed, there was no significant difference according to the level of SIRT4 gene expression. Patient numbers are shown in Table 3.15 and the recurrence data in (Figure 3.62). Time to recurrence was shorter in patients with tumours that expressed low levels of SIRT4 (n = 11, 8.87 years, 95% C.I. 4.49-13.25) when compared to those whose tumours expressed low levels of SIRT4 (n = 25, 11.78 years, 95% C.I. 9.57 -13.99; Figure 3. 62). However the difference was not significant (Kaplan-Meier p = 0.125).

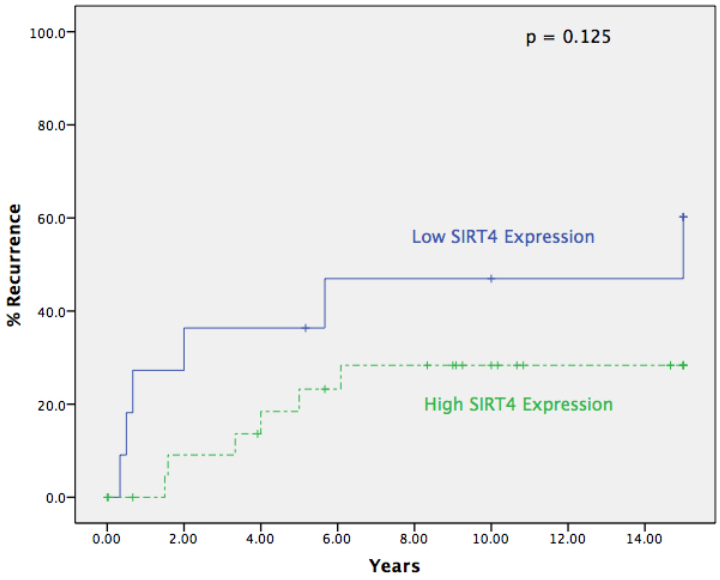

Figure 3.62: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT4 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed a non-significant decrease in the survival period in patients with tumours that express low levels of SIRT4 (n = 11, 8.87 years, 95% C.I. 4.49-13.25) when compared to those whose tumours express low levels of SIRT4 (n = 25, 11.78 years, 95% C.I. 9.57 -13.99, Kaplan-Meier p = 0.125).

3.4.5.2.3 Recurrence analysis in ER^{+ve} patients according to SIRT4 levels.

Further analysis of recurrence within the subgroup of patients with $ER^{+ve)}$ tumours showed a significant difference in time to recurrence according to the level of SIRT4 gene expression. Patient numbers are shown in (Table 3.15). Time to recurrence was significantly shorter in patients with tumours that expressed low levels of SIRT4 (n = 9, 5.79 years, 95% C.I. 1.59-9.99) when compared to those whose tumours expressed high levels of SIRT4 (n = 22, 11.29 years, 95% C.I. 8.57 -14.01, Kaplan-Meier p = 0.023, Figure 3.63).

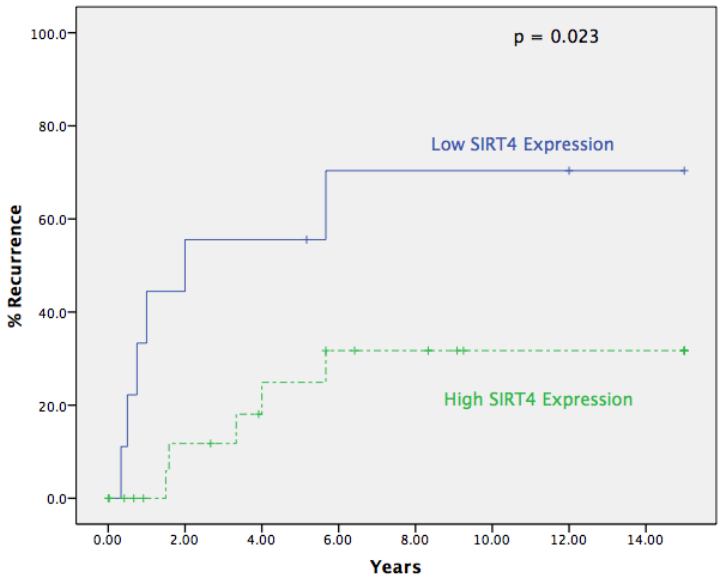

Figure 3.63: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT4 expression in patients with ER^{+ve} tumours.

High and low SIRT4 expression groups were determined as above or below the lower tertile cutoff value. The recurrence curve showed a significant decrease in the time to recurrence in patients with tumours that express low levels of SIRT4 (n = 9, 5.79 years, 95% C.I. 1.59-9.99) when compared to those whose tumours express high levels of SIRT4 (n = 22, 11.29 years, 95% C.I. 8.57 -14.01, Kaplan-Meier p = 0.023).

3.4.6 Conclusion.

This study investigated the relative transcriptional expression levels of the SIRT4 gene in (25) normal, (72) non-malignant and (69) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT4 as the data distributions were positively skewed in all patient groups.

The data for SIRT4 gene expression variation between breast biopsies showed that the SIRT4 levels were significantly lower in malignant group when compared to the non-malignant group with a similar age distribution. However, the expression level of SIRT4 did not show any significant difference when the malignant group was compared with a younger normal cohort.

There was a significant association between increasing relative transcription levels of SIRT4 and chronological age.

SIRT4 gene expression did not show significant correlation with patient survival, either in the entire patient set, or in a patient group selected by NPI, or ER^{+ve} status, to have an intermediate clinical prognosis. SIRT4 gene expression did not show significant correlation with tumour recurrences, either in the entire patient set, or in a patient group selected by NPI to have an intermediate clinical prognosis. However, there was a significant difference in the recurrence time in ER^{+ve} breast cancer patients, with shorter recurrence time associated with lower SIRT4 expression. There was a marginally significant correlation between decreased SIRT4 gene expression level and the presence of metastasis

The data for SIRT4 gene expression variation between breast biopsies showed that the malignant group differs significantly from the non-malignant group of similar age. However, the expression level of SIRT4 did not show any significant difference when the malignant group was compared with a younger normal cohort.

3.5 The relative transcriptional expression levels of SIRT5 in breast biopsies.

3.5.1 The relative transcriptional expression levels of SIRT5 and patients age.

The relative transcriptional expression levels of the SIRT5 gene were determined in 24 normal biopsies, 69 non-malignant and 69 malignant patient biopsies (Table 3.16).

Table 3.16 Patient biopsies used in the SIRT5 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT5 Expression	Normal	24	92.30%	2	7.70%	26	100.00%
	Non-Malignant	69	88.50%	9	11.50%	78	100.00%
	Malignant	69	94.50%	4	5.50%	73	100.00%

The correlation between the level of SIRT5 relative transcriptional expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3.64 a). The median age of this patient group was 51years (20 years - 84 years). This data showed that there was no correlation between the relative transcriptional levels of SIRT5 and chronological age (Spearman's rank correlation coefficient; p = 0.129; Figure 3. 64b).

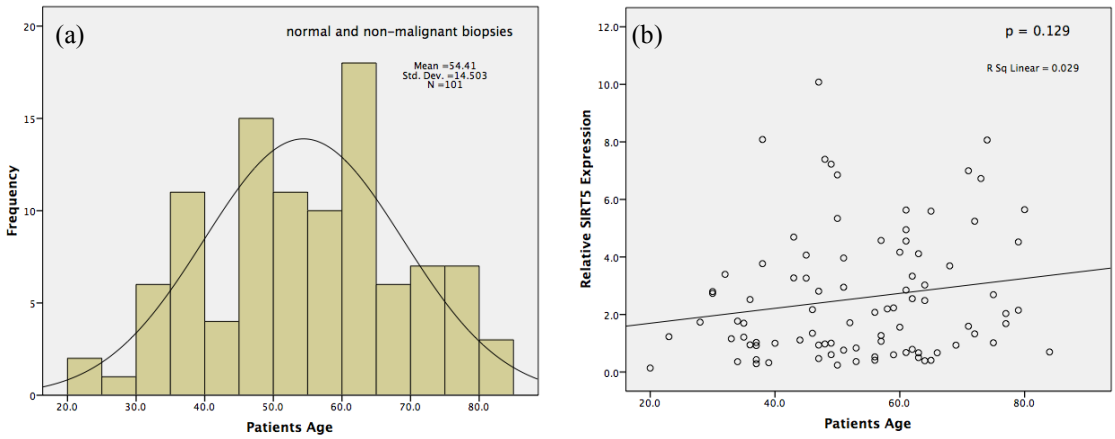

Figure 3.64: (a) Histogram shows the distribution of patient's age patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT5 and patient's age in patient group that combines both normal and non-malignant patients (Spearman test, p = 0.129).

3.5.2 Data distribution of the relative transcriptional expression levels of SIRT5.

The distribution of the relative transcriptional gene expression of SIRT5 was investigated in the three different studied biopsy types (Figure 3.65). The histograms of the distribution of the relative transcriptional gene expression of SIRT5 showed that the range of the SIRT5 gene expression values was not normally distributed; instead the SIRT5 gene expression distribution was positive skewed in all groups (Figure 3. 65). Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT5.

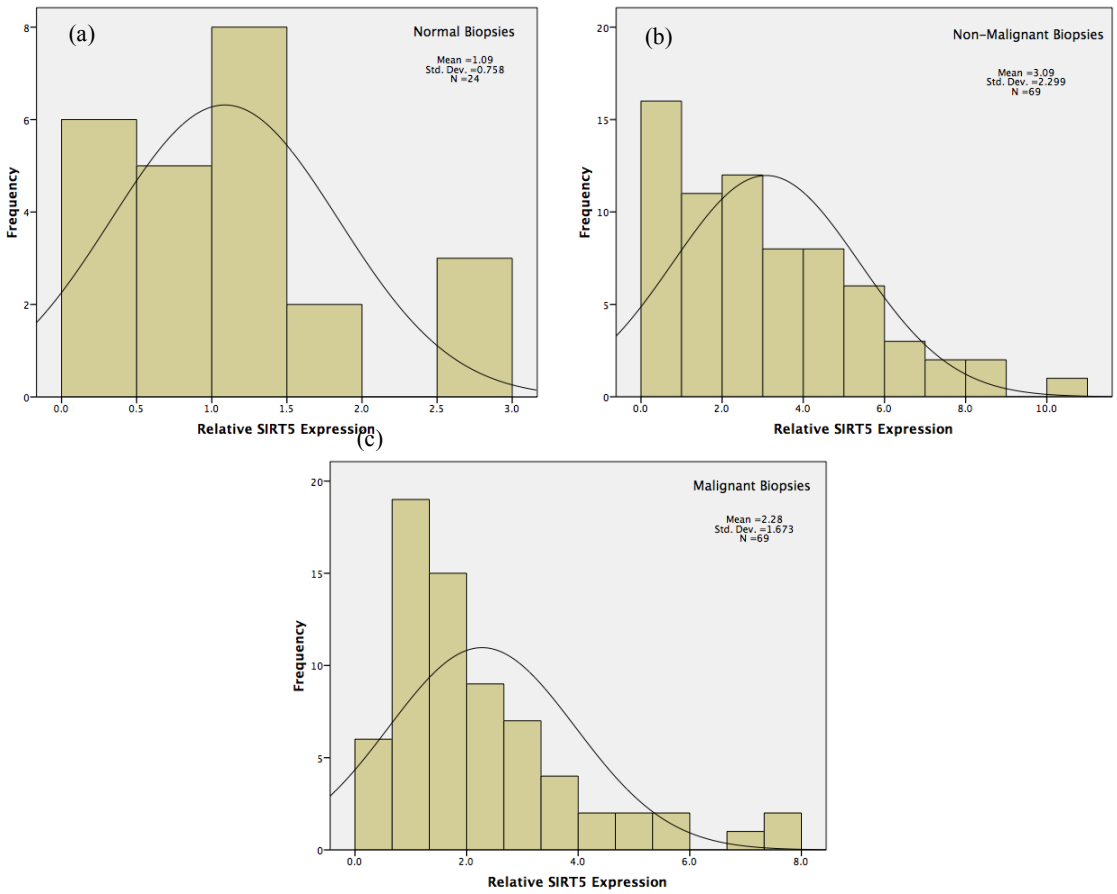

Figure 3.65: Distribution of the relative transcriptional gene expression of SIRT5 in Three different groups.

(a) Relative transcriptional gene expression of SIRT5 in normal breast biopsies. (b) Relative transcriptional gene expression of SIRT5 in non-malignant breast biopsies. (c) Relative transcriptional gene expression of SIRT5 in malignant breast biopsies.

3.5.3 Comparison of the relative transcriptional expression levels of SIRT5 between patient groups

The level of SIRT5 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT5 was significantly different between the three groups of breast biopsies ($p < 0.001$, Figure 3.66). This experiment showed that normal samples have significantly lower levels of SIRT5 gene expression compared to both malignant and non-malignant samples ($p < 0.001$, Figure 3. 66).

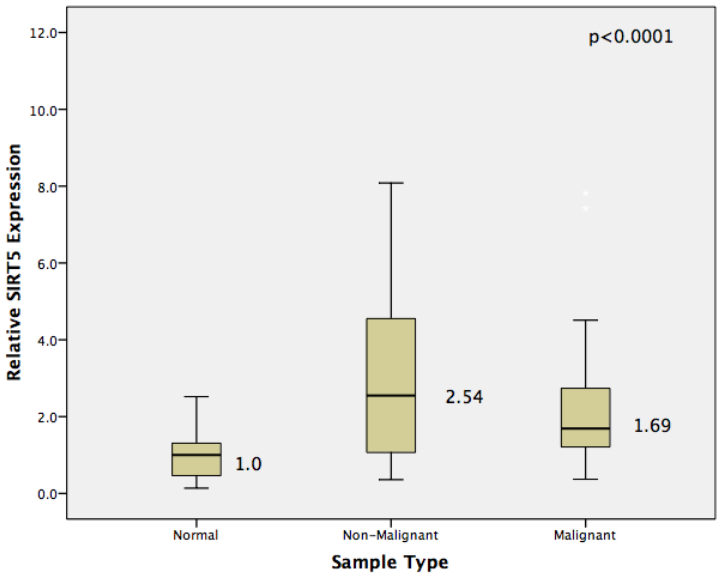

Figure 3.66: Box plot showing the transcriptional expression of SIRT5 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

The median value for each patient group is shown to the right of each box plot ($p < 0.001$).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3. 6) showed that the normal group differed significantly in SIRT5 gene expression from both malignant and non-malignant groups (normal versus non-malignant $p < 0.001$, normal versus malignant; $p < 0.001$). However, at the 5% level of significance the data showed no significant difference between the malignant and non-malignant groups ($p = 0. 072$).

3.5.4 Comparison of the relative transcriptional expression levels of SIRT5 according to histopathological markers.

The relationships between SIRT5 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.5.4.1 Comparison of the relative transcriptional expression levels of SIRT5 according to tumour grade, tumour size, nodal status, oestrogen receptor status and lymphovascular invasion

Analysis of the data for the relationship between SIRT5 gene expression and tumour grade, tumour size, nodal status, oestrogen receptor status and lymphovascular invasion all showed no significant differences in the relative transcriptional expression levels of SIRT5 between any categories when compared together (Figure 3. 67 a, b, c, d and e). Table 3.6 shows the Kruskal-Wallis test p values for all comparisons.

3.5.5 The relationship of the relative transcriptional expression levels of SIRT5 in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT5 relative gene expression, the patients were grouped in tertiles according to the level of SIRT5 gene expression. In analyses shown below, the two tertiles with lower SIRT5 gene expression levels have been combined and are compared to the highest level of SIRT5 gene expression tertile group. Table 3.17 and Table 3.18 show the numbers of patients included in each group.

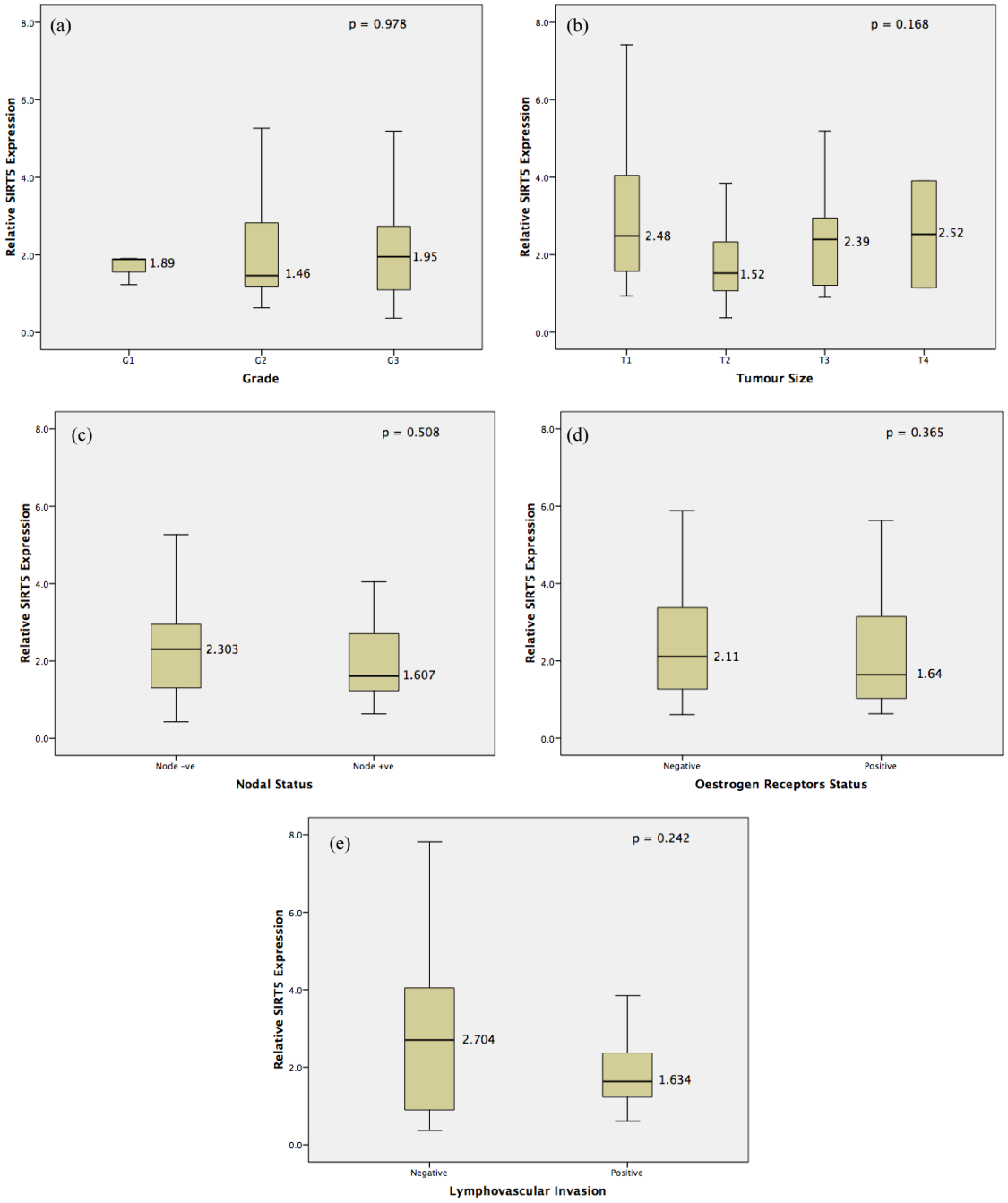

Figure 3.67: Box plots showing the relative transcriptional expression of SIRT5 in breast cancer biopsies by known prognostic markers:

(a) tumour grade, n = 3 for G1, n = 32 for G2, and n = 29 for G3 (Mann Whitney test p = 0.978). (b) tumour size, n = 10 for T1, n = 43 for T2 , n = 13 for T3 and n = 2 for T4 (Kruskal Wallis Test, p = 0.168), (c) nodal status, n = 18 for node negative and n = 42 for node positive (Mann Whitney test p = 0.508. (d) oestrogen receptor status, n = 20 for ER^{-ve} and n = 31 for ER^{+ve} (Mann Whitney test p = 0.365. e) Lymphovascular invasion (LV) n = 17 for LV^{-ve} and n = 28 for LV^{+ve} (Mann Whitney test p = 0.242). The median value for each patient group is shown right of each box.

3.5.5.1 SIRT5 and survival

3.5.5.1.1 Survival analysis in entire patient cohort according to SIRT5 levels.

Analysis of the survival data for the entire patient group using the Kaplan-Meier test showed no significant difference in survival period when all patients were included (Table 3.17). The survival period was not significantly shorter in patients with tumours that expressed low levels of SIRT5 (n = 43, 10.48 years, 95% C.I. 8.7 -12.26) when compared to those whose tumours expressed high levels of SIRT5 (n = 23, 12.28 years, 95% C.I. 10.24 – 14.31, Kaplan-Meier p = 0.305; Figure 3. 68).

Table 3.17: Number of breast cancer patient and cancer specific deaths of all breast cancer patient groups included in the Kaplan-Meier survival analysis.

SIRT5 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT5 gene expression	43	15	22	6	18	7
High SIRT5 gene expression	23	5	12	3	11	3
Overall	66	20	34	9	29	10

Figure 3.68: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT5 expression.

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a non significant decrease in tumours that express low levels of SIRT5 (n = 43, 10.48 years, 95% C.I. 8.7 -12.26) when compared to those whose tumours express high levels of SIRT5 (n = 23, 12.28 years, 95% C.I. 10.24 – 14.31, Kaplan-Meier p = 0.305).

3.5.5.1.2 Survival analysis in NPI selected patients according to SIRT5 levels.

Analysis of the survival data for the NPI-selected patient group (Table 3.17) using the Kaplan-Meier test did not show any significant difference in the survival period in patients with tumours that expressed low levels of SIRT5 (n = 22, 12.11 years, 95% C.I. 10.11 - 14.1) when compared to those whose tumours expressed high levels of SIRT5 (n = 12, 12.25 years, 95% C.I. 9.695 – 14.8, Kaplan-Meier p = 0.983; Figure 3. 69).

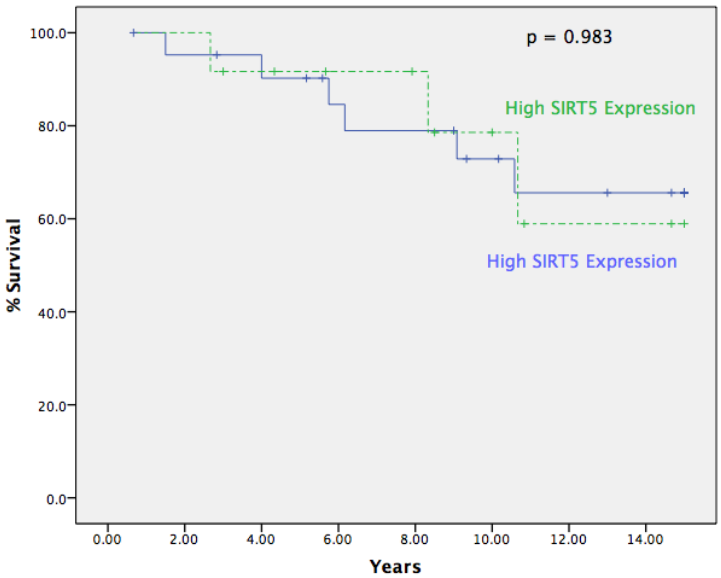

Figure 3.69: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT5 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows insignificant difference in the survival period in patients with tumours that express low levels of SIRT5 (n = 22, 12.11 years, 95% C.I. 10.11 - 14.1) when compared to those whose tumours express high levels of SIRT5 (n = 12.25 years, 95% C.I. 9.695 – 14.8, Kaplan-Meier p = 0.983).

3.5.5.1.3 Survival analysis in ER^{+ve} selected patients according to SIRT5 levels.

Analysis of the survival data for the ER^{+ve} patient group (Table 3. 17) using the Kaplan-Meier test showed no significant difference in the survival period in patients with tumours that expressed low levels of SIRT5 (n = 18, 9.53 years, 95% C.I. 6.59-12.97) when compared to those whose tumours expressed high levels of SIRT5 (n = 11, 11.33 years, 95% C.I. 8.17 – 14.5, Kaplan-Meier p = 0.538, Figure 3.70).

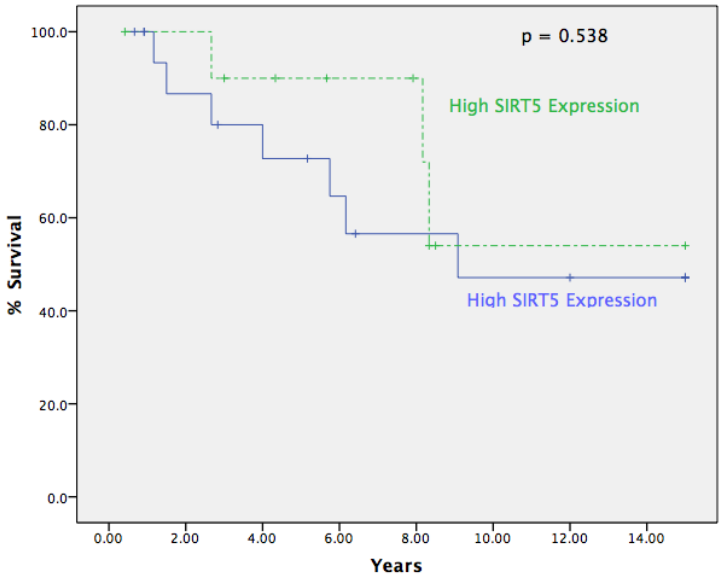

Figure 3.70: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT5 expression in patients with ER^{+ve} tumours.

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows non significant difference in the survival period in patients with tumours that express low levels of SIRT5 (n = 18, 9.53 years, 95% C.I. 6.59-12.97) when compared to those whose tumours express high levels of SIRT5 (n = 11, 11.33 years, 95% C.I. 8.17 – 14.5, Kaplan-Meier p = 0.538.

3.5.5.2 SIRT5 and Recurrence.

3.5.5.2.1 Recurrence analysis in entire patient cohort according to SIRT5 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, in all breast cancer patients, patient numbers are shown in (Table 3.18). There was no significant differences in the recurrence interval between patients with tumours that expressed low levels of SIRT5 (n = 43, 10.61 years, 95% C.I. 10.61-12.63) when compared to those whose tumours express high levels of SIRT5 (n = 23, 12.32 years, 95% C.I. 10.0 -14.63, Kaplan-Meier p = 0.272, Figure 3.71).

Table 3.18: Number of breast cancer patient and tumour recurrences of all breast cancer patient groups included in the Kaplan-Meier survival analysis.

SIRT5 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT5 gene expression	43	14	23	10	19	8
High SIRT5 gene expression	23	4	12	2	11	3
Overall	66	18	33	12	29	11

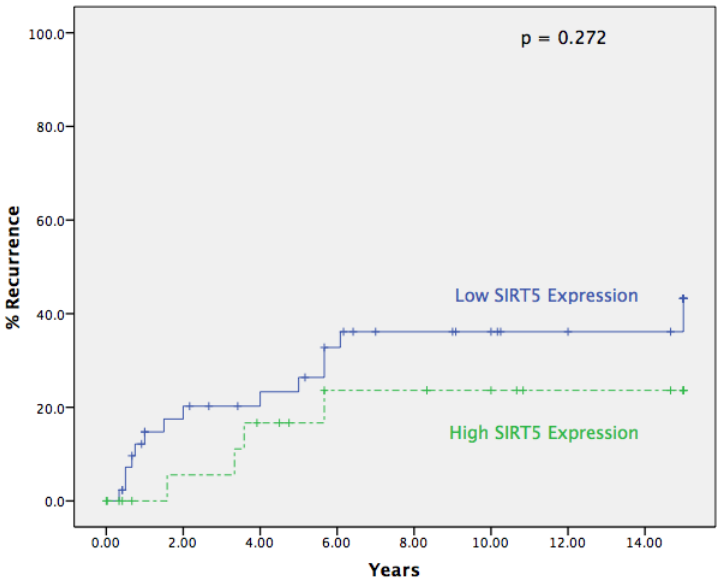

Figure 3.71: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT5 expression.

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a non significant difference in the time to recurrence in patients with tumours that express low levels of SIRT5 (n = 43, 10.61 years, 95% C.I. 10.61-12.63) when compared to those whose tumours express high levels of SIRT5 (n = 23, 12.32 years, 95% C.I. 10.0 -14.63, Kaplan-Meier p = 0.272.

3.5.5.2.2 Recurrence analysis in NPI selected patients according to SIRT5 levels.

When tumour recurrence among the NPI-selected patient group was analysed (Table 3. 18), there was no significant difference according to the level of SIRT5 gene expression in patients with tumours that expressed low levels of SIRT5 (n = 23, 10.04 years, 95% C.I. 7.35-12.72) when compared to those whose tumours expressed high levels of *SIRT5* (n = 12, 12.49 years, 95% C.I. 9.37 -15.61, Kaplan-Meier p = 0.287; Figure 3.72).

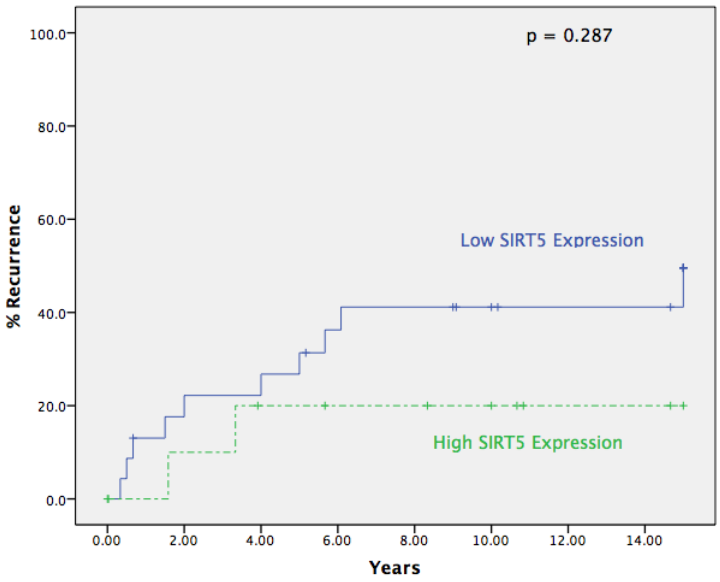

Figure 3.72: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT5 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a non significant difference in the time to recurrence period in patients with tumours that express low levels of SIRT5 (n = 23, 10.04 years, 95% C.I. 7.35-12.72) when compared to those whose tumours express high levels of SIRT5 (n = 12, 12.49 years, 95% C.I. 9.37 -15.61, Kaplan-Meier p = 0.287).

3.5.5.2.3 Recurrence analysis in ER^{+ve} patients according to SIRT5 levels.

Further analysis of recurrence within the subgroup of patients with ER^{+ve} tumours failed to reveal any significant difference in time to recurrence between patients with tumours that expressed low levels of SIRT5 (n = 19, 8.82 years, 95% C.I. 5.66 -11.98) when compared to those whose tumours expressed high levels of SIRT5 (n = 11, 10.46 years, 95% C.I. 6.41-14.51, Kaplan-Meier p = 0.531, Figure 3.73). Patient numbers for each group are shown in Table 3.18.

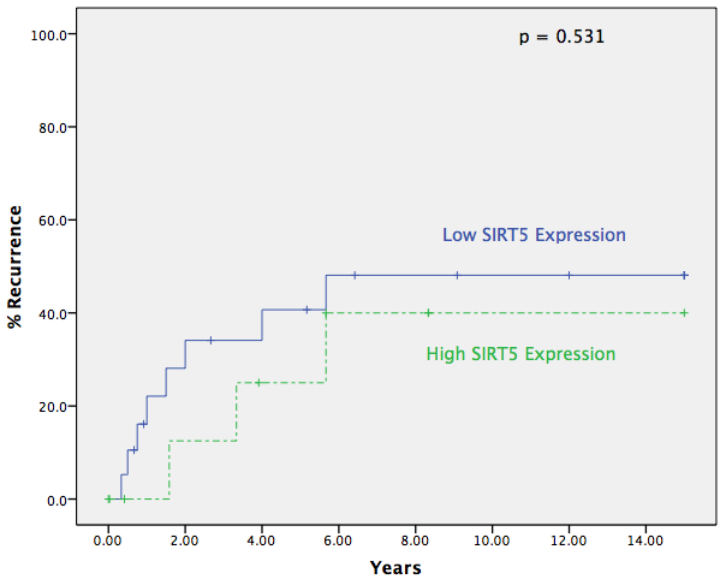

Figure 3.73: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT5 expression in patients with ER^{+ve} tumours.

High and low SIRT5 expression groups were determined as above or below the upper tertile cutoff value. The recurrence curve showed a insignificant difference in the time to recurrence in patients with tumours that express low levels of SIRT5 (n = 19, 8.82 years, 95% C.I. 5.66 -11.98) when compared to those whose tumours express high levels of SIRT5 (n = 11, 10.46 years, 95% C.I. 6.41-14.51, Kaplan-Meier p = 0.531).

3.5.6 Conclusion

This study investigated the relative transcriptional expression levels of the SIRT5 gene in (25) normal, (69) non-malignant and (69) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT5 as the data distributions were positively skewed in all patient groups.

The data for SIRT5 gene expression variation between breast biopsies did not show a clear significant difference between the malignant and non-malignant groups of similar age, although there may be a trend towards decreased expression associated with malignant changes. Interestingly, the expression level of SIRT5 did show significant differences when the normal group was compared with both non-malignant and malignant groups.

SIRT5 gene expression did not show significant correlation with patient survival, or tumour recurrence, either in the entire patient set, or in a patient group selected by NPI, or ER^{+ve} status to have an intermediate clinical prognosis.

3.6 The relative transcriptional expression levels of SIRT6 in breast biopsies.

3.6.1 The relative transcriptional expression levels of SIRT6 and patients age.

The relative transcriptional expression levels of SIRT6 gene were determined in 25 normal biopsies, (73) non-malignant and (70) malignant (Table 3. 19).

Table 3.19: Patient biopsies used in the SIRT6 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT6 Expression	Normal	25	96.20%	1	3.80%	26	100.00%
	Non-Malignant	73	93.60%	5	6.40%	78	100.00%
	Malignant	70	95.90%	3	4.10%	73	100.00%

The correlation between the level of SIRT6 relative transcriptional expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3.74 a). The median age of this patient group was 51years (20 years -84 years). This data showed that there was no significant correlation between the relative transcriptional levels of SIRT6 and chronological age (Spearman's rank correlation coefficient; p = 0.153; Figure 3.74 b).

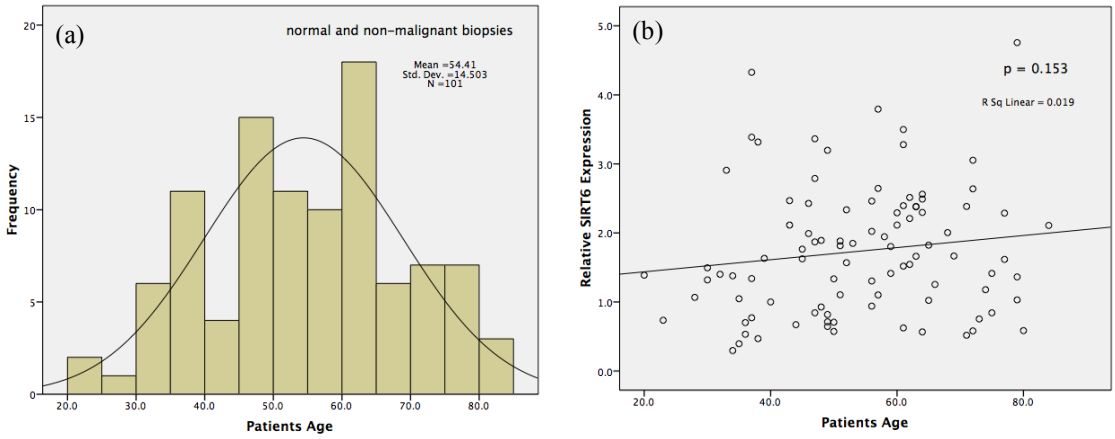

Figure 3.74: (a) Distribution of patient's age patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT6 and patient's age in patient group that combines both normal and non-malignant patients (Spearman test, p = 0.153).

3.6.2 Data distribution of the relative transcriptional expression levels of SIRT6.

The distribution of the relative transcriptional gene expression of SIRT6 was investigated in the three different studied biopsy types, (Figure 3.75). The histograms of the distribution of the relative transcriptional gene expression of SIRT6 showed that the range of the SIRT1 gene expression values was not normally distributed; instead the SIRT6 gene expression distribution was positively skewed in normal and non-malignant groups. However, SIRT6 gene expression data was normally distributed in the malignant group. (Figure 3.75) Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT6.

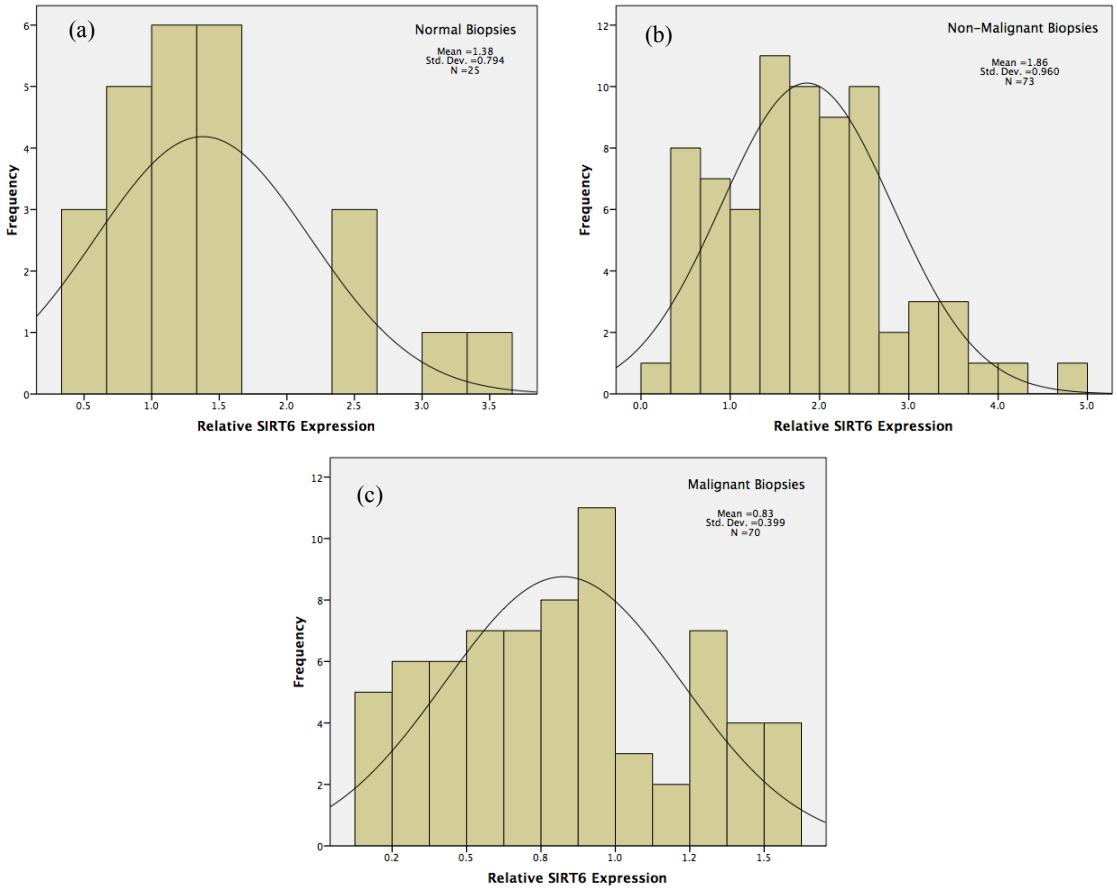

Figure 3.75: Distribution of the relative transcriptional gene expression of SIRT6 in Three different groups.

(a) Relative transcriptional gene expression of SIRT6 in normal breast biopsies. (b) Relative transcriptional gene expression of SIRT6 in non-malignant breast biopsies. (c) Relative transcriptional gene expression of SIRT6 in malignant breast biopsies.

3.6.3 Comparison of the relative transcriptional expression levels of SIRT6 between patient groups.

First, the level of SIRT6 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT6 was significantly different between the three groups of breast biopsies ($p < 0.001$, Figure 3.76). This experiment showed that malignant samples have significantly lower levels of SIRT6 gene expression compared to both normal and non-malignant samples ($p < 0.001$, Figure 3.76).

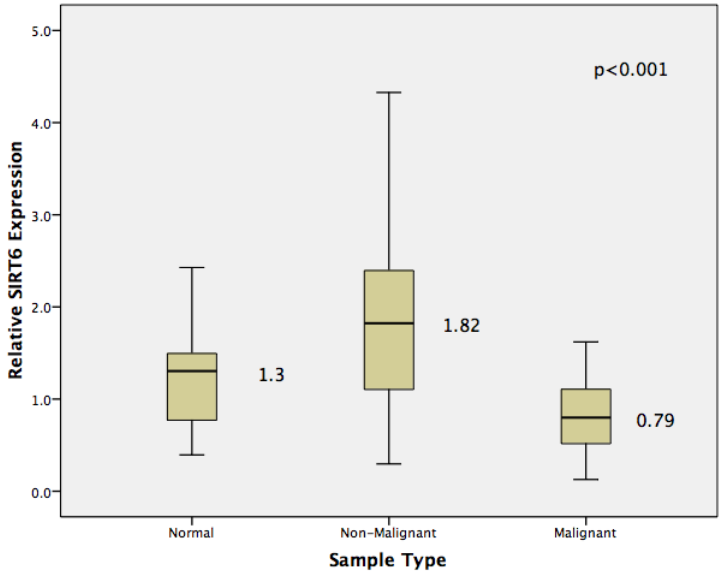

Figure 3.76: Box plot showing the transcriptional expression of SIRT6 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

The median value for each patient group is shown in the clear box to the right of each box plot ($p < 0.001$).

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6) showed that the malignant groups had significantly lower levels of SIRT6 gene expression when compared with both ($p < 0.001$) normal and non- malignant ($p < 0.001$) groups. However, there was a significant increase in the levels of SIRT6 gene expression in the non-malignant when compared with normal breast biopsies ($p = 0.019$, Figure 3.76, Table 3.6).

3.6.4 Comparison of SIRT6 gene transcription levels according to histopathological markers

The relationships between SIRT6 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.6.4.1 Comparison of the relative transcriptional expression levels of SIRT6 according to tumour grades.

Analysis of the data for the relationship between SIRT6 gene expression and tumour grade showed a significant difference in the relative transcriptional expression levels of SIRT6 between all three tumour grades when compared together (Kruskal-Wallis test p = 0.002) (Figure 3.77). The relative transcriptional expression of SIRT6 decreases with increasing severity (higher tumour grade) of tumour.

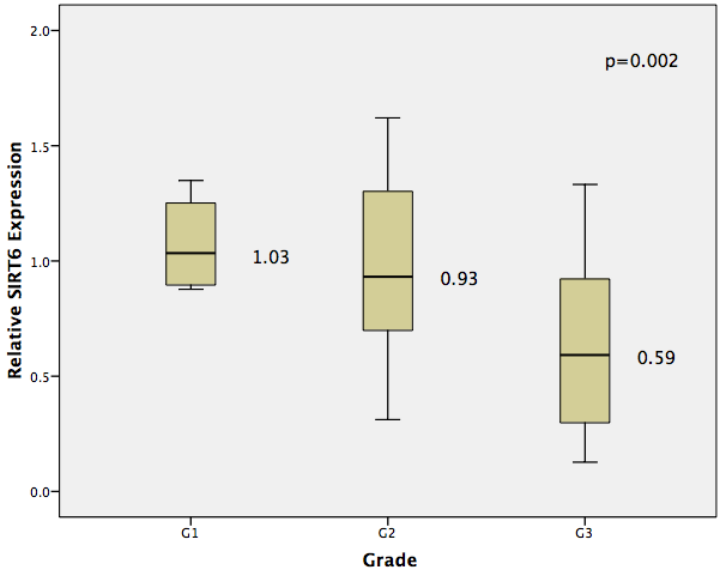

Figure 3.77: Box plot showing the relative transcriptional expression of in breast cancer biopsies by tumour grade.

n = 4 for grade 1, 32 for grade 2 and 29 for grade 3 *(*Kruskal Wallis Test, *p = 0.002)*. The median value for each patient group is shown in the clear box to the right of each box plot.

Pairwise comparisons of the three groups using the Mann-Whitney test (Table 3.6) showed that grade 3 tumours groups differed significantly in SIRT6 gene expression from both grade 1 (p = 0.04) and grade 2 tumours (p = 0.001), whereas there was no significant difference between grade 1 and grade 2 tumours (p = 0.610; Figure 3. 77).

3.6.4.2 Comparison of the relative transcriptional expression levels of SIRT6 according to tumour size, nodal status, oestrogen receptor status and lymphovascular invasion

Analysis of the data for the relationship between SIRT6 gene expression and tumour size, nodal status, ER status and lymphovascular invasion all showed no significant difference in the relative transcriptional expression levels of SIRT6 between any categories when compared together (Figure 3. 78 a, b, c and d). Table 3.6 shows the Kruskal-Wallis test p values for all comparisons.

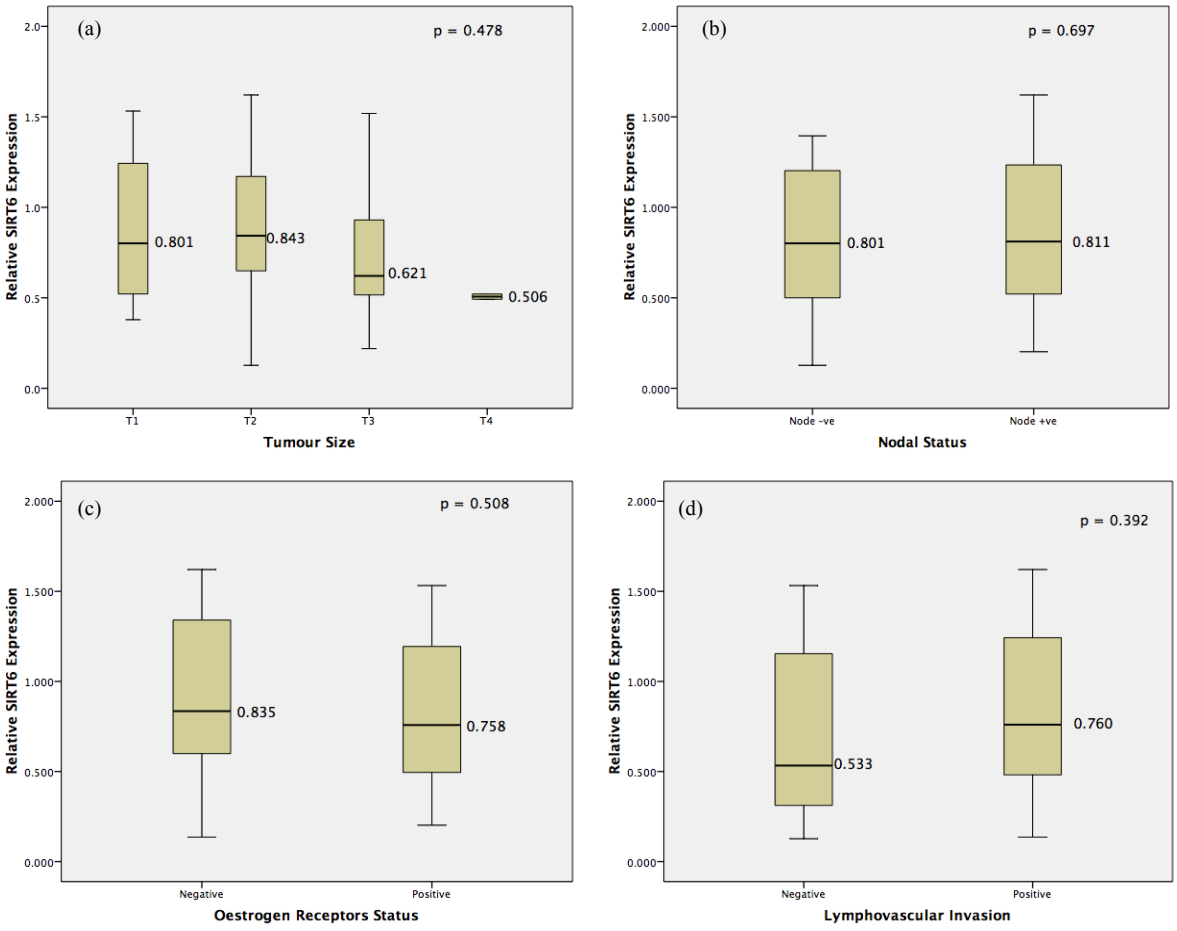

Figure 3.78 : Box plots showing the relative transcriptional expression of SIRT6 in breast cancer biopsies by known prognostic markers:

(a) tumour size, n = 11 for T1,n = 43 for T2 , n = 13 for T3 and n = 2 for T4 *(*Kruskal Wallis Test, *p = 0.478).* (b) nodal status n = 19 for node negative and n = 42 for node positive, *(*Kruskal Wallis Test p = 0.697), (c) oestrogen receptor status n = 19 for ER negative and n = 32 for ER positive (Kruskal Wallis Test, p = 0.508*). (d) lymphovascular invasion, n = 18 for negative lymphovascular invasion and n = 28 for positive negative lymphovascular invasion (Kruskal Wallis Test, p = 0.392*). The median value for each patient group is shown in the clear box to the right of each box plot.

3.6.5 The relationship of the relative transcriptional expression levels of SIRT6 in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT6 relative gene expression, the patients were grouped in tertiles according to the level of SIRT6 gene expression. In analyses shown below, the two tertiles with lower SIRT6 gene expression have been combined and are compared to the highest levels of SIRT6 gene expression tertile group. Table 3.20 and Table 3. 22 show the numbers of patients included in each group.

3.6.5.1 SIRT6 and survival.

3.6.5.1.1 Survival analysis in entire patient cohort according to SIRT6 levels.

Analysis of the survival data for the entire patient group (Table 3. 20) using the Kaplan-Meier test showed that the mean survival period was significantly shorter in patients with lower SIRT6 expression (n = 43, 9.67 years, 95% C.I. 7.8 -11.5) when compared with those with higher SIRT6 expression (n = 24, 13.6 years, 95% C.I. 12.35 -15.04, Kaplan-Meier, p = 0.009, Figure 3. 79). Furthermore, Cox-regression analysis of the hazard ratios revealed that patients with decreased tumour expression for SIRT6 were 1.5 times (95% CI 1.31-15.41, p = 0.017) more likely to die as a consequence of breast cancer disease than those patients with high levels of SIRT6.

Table 3.20: Number of breast cancer patient and cancer specific deaths of all patient groups included in the Kaplan-Meier survival analysis.

SIRT6 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT6 gene expression	43	17	17	8	19	10
High SIRT6 gene expression	24	3	17	1	11	0
Overall	67	20	34	9	30	10

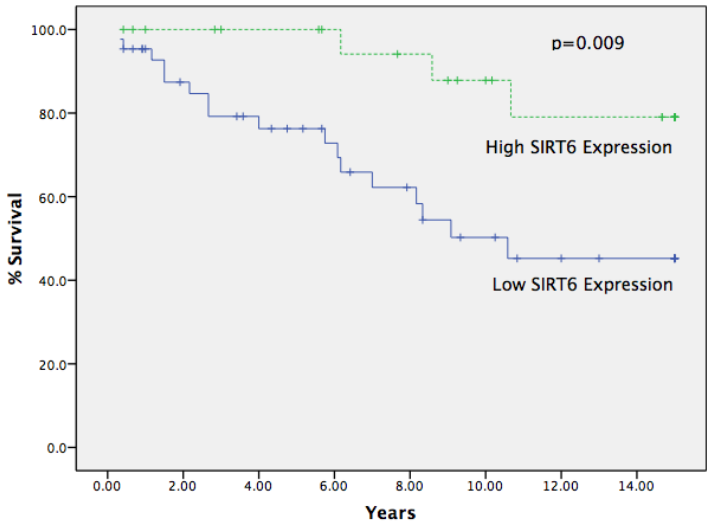

Figure 3.79: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT6 expression.

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a significant decrease in the survival period for patients with tumours that express low levels SIRT6 (n = 43, 9.67 years, 95% C.I. 7.8 -11.5) when compared to those whose tumours express high levels of SIRT6 (n = 24, 13.6 years, 95% C.I. 12.35 -15.04, Kaplan-Meier survival analysis, p = 0.009).

Multivariate Cox-regression analysis demonstrated that the relative transcriptional level of SIRT6 gene was independent of tumour size, grade, nodal status, ER status, lymphovascular invasion status, and Nottingham Prognostic Index (NPI) in influencing survival ($p = 0.035$, Table 3.21).

Table 3.21: Multivariate Cox-regression stepwise analysis for breast cancer specific survival for SIRT6 and prognostic factors.

p values, Hazard ratio (HR) for death and confidence interval (CI) for the HR for SIRT6 and each prognostic factor are displayed in the table.

		Sig.	HR	95.0% CI Lower	Upper
Step 1	SIRT6	0.028	-3.034	0.003	0.716
	Tumour Grade	0.335	-1.12	0.034	3.179
	Tumour Size	0.183	1.234	0.558	21.152
	N	0.135	1.928	0.549	86.074
	ER	0.059	-2.989	0.002	1.118
	LV	0.362	-1.267	0.019	4.283
	NPI	0.547	0.868	0.141	40.118
Step 2	SIRT6	0.024	-2.859	0.005	0.685
	Tumour Grade	0.445	-0.621	0.109	2.643
	Tumour Size	0.097	1.431	0.77	22.705
	N	0.007	2.47	1.94	72.124
	ER	0.042	-2.493	0.007	0.915
	LV	0.444	-0.886	0.043	3.984
Step 3	SIRT6	0.034	-2.502	0.008	0.824
	Tumour Size	0.123	1.295	0.704	18.932
	N	0.011	2.412	1.734	71.832
	ER	0.055	-2.28	0.01	1.049
	LV	0.535	-0.686	0.058	4.394
Step 4	SIRT6	0.038	0.089	0.009	0.879
	Tumour Size	0.05	4.645	1.001	21.562
	N	0.014	10.672	1.625	70.096
	ER	0.034	0.163	0.03	0 .874

3.6.5.1.2 Survival analysis in NPI selected patients according to SIRT6 levels.

Analysis of the survival data in patients with (NPI between 3.4-5.4, Table 3.20) demonstrated that lower SIRT6 expression remained significantly associated with shorter survival. Mean survival period was significantly shorter (n = 17, 9.87 years, 95% C.I. 7.37-12.37) in patients with tumours that expressed low levels of SIRT6 when compared with those with high level of SIRT6 (n = 17, 14.5 years, 95% C.I. 13.6-15.4, Log Rank, p=0.004, Figure 3. 80).

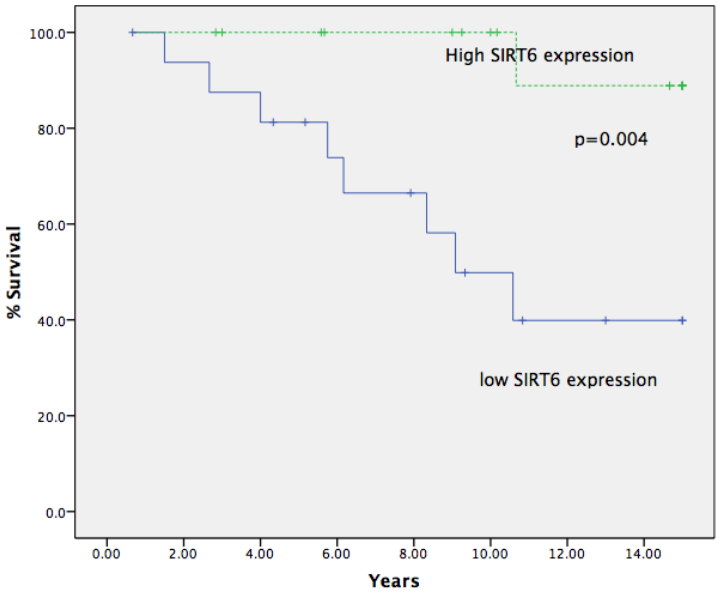

Figure 3.80: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT6 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows a significant decrease in the survival period for patients with tumours that express low levels SIRT6 (n = 17, 9.87 years, 95% C.I. 7.37-12.37) when compared to those whose tumours express high levels of SIRT6 (n = 17, 14.5 years, 95% C.I. 13.6-15.4, Kaplan-Meier, p=0.004).

3.6.5.1.3 Survival analysis in ER^{+ve} patients according to SIRT6 levels.

Analysis of the cancer specific survival data in patients with ER^{+ve} tumours (Table 3.20) showed significant differences in the survival period between patients with tumours that expressed high levels of SIRT6 (n = 19) when compared to those whose tumours expressed low levels of SIRT6 (n = 11, Kaplan-Meier p = 0.002, Figure 3.81). The mean survival time could not be calculated for patient with high SIRT6 gene expression because of the small number of patients and lack of complete (15 years) follow up data.

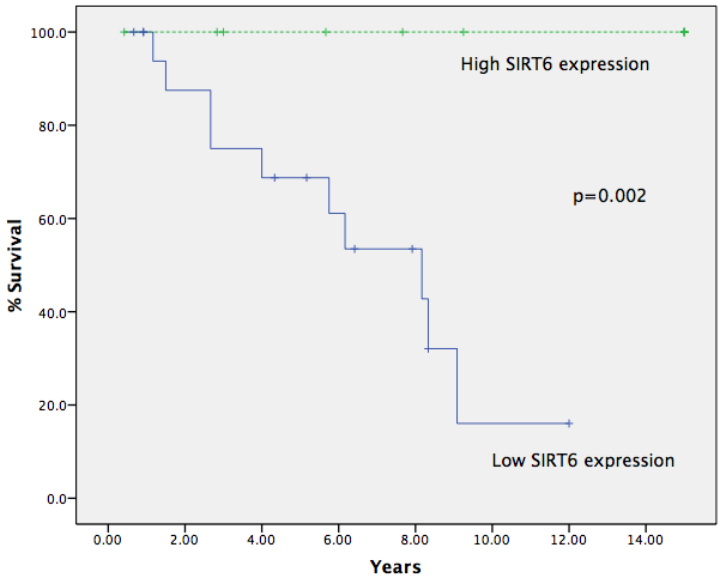

Figure 3.81: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT1 expression in patients with ER^{+ve}.

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The survival curve shows significant difference in the survival period for patients with tumours that express high levels SIRT6 (n = 19) when compared to those whose tumours express low levels of SIRT6 (n = 11; Kaplan-Meier p = 0.002).

3.6.5.2 SIRT6 and recurrence.

3.6.5.2.1 Recurrence analysis in entire patient cohort according to SIRT6 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, and showed a statistically non-significant decrease in the recurrence interval in patients with high levels of SIRT6 gene expression when all patients were included. Patient numbers are shown in Table 3.22. Time to recurrence was statistically non significantly shorter in patients with tumours that expressed low levels of SIRT6 (n = 43, 10.45 years, 95% C.I. 8.28-12.62) when compared to those whose tumours expressed high levels of *SIRT6* (n = 24, 12.24 years, 95% C.I. 10.10 -14.38, Kaplan-Meier p = 0.178, Figure 3.82).

Table 3.22 Number of breast cancer patient and tumour recurrences of all patient groups included in the Kaplan-Meier recurrence analysis.

SIRT6 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT6 gene expression	43	13	18	8	20	8
High SIRT6 gene expression	24	5	17	4	11	3
Overall	67	18	35	12	31	11

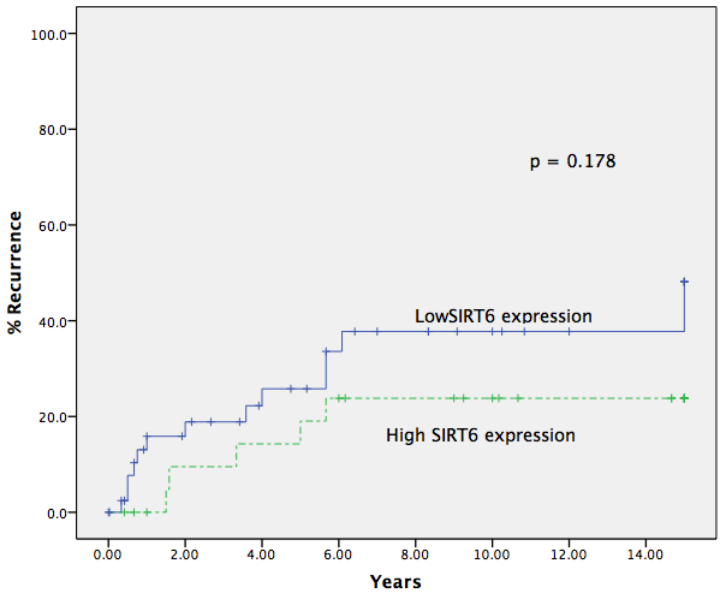

Figure 3.82: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT6 expression.

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed that time to recurrence was non significantly shorter in patients with tumours that expressed low levels of SIRT6 (n = 43, 10.45 years, 95% C.I. 8.28-12.62) when compared to those whose tumours express high levels of SIRT6 (n = 24, 12.24 years, 95% C.I. 10.10 -14.38, Kaplan-Meier p = 0.178).

3.6.5.2.2 Recurrence analysis in NPI selected patients according to SIRT6 levels.

When tumour recurrence among patients with moderately good prognosis (NPI between 3.4 and 5.4; Table 3.22), was analysed according to the level of SIRT6 gene expression there was a trend toward significant in the time to recurrence. Kaplan-Meier recurrence analysis showed that time to recurrence was shorter in patients with tumours that expressed low levels of SIRT6 (n = 18, 9.05 years, 95% C.I. 5.59-12.5) when compared to those whose tumours expressed high levels of SIRT6 (n = 17, 12.14 years, 95% C.I. 9.67 -14.6, Kaplan-Meier p = 0.067, Figure 3.83).

3.6.5.2.3 Recurrence analysis in ER^{+ve} selected patients according to SIRT6 levels.

Further analysis of recurrence within the subgroup of patients with ER^{+ve} tumours (Table 3.22) failed to reveal any significant difference in time to recurrence between patients with tumours that expressed low levels of SIRT6 (n = 20, 6.95 years, 95% C.I. 4.48-9.42) when compared to those whose tumours expressed high levels of *SIRT6* (n = 11, 11.14 years, 95% C.I. 7.47, 14.8, Kaplan-Meier p = 0.282, Figure 3.84).

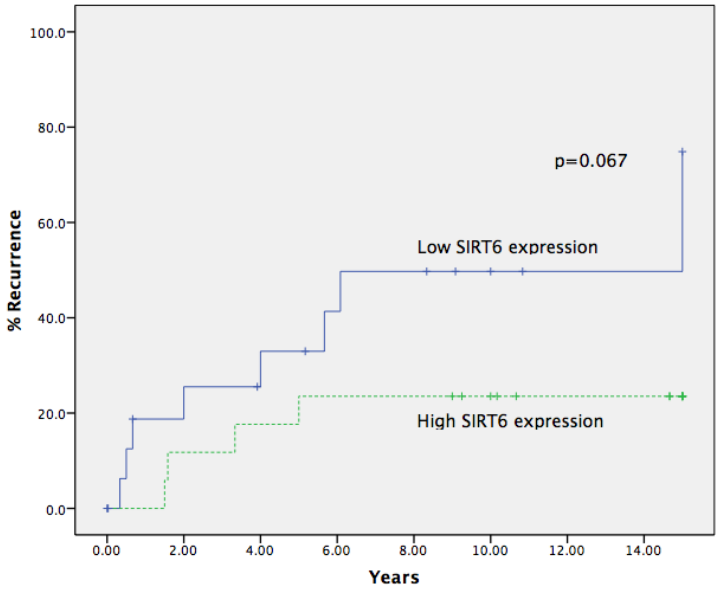

Figure 3.83: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT6 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed that time to recurrence was shorter in patients with tumours that expressed low levels of SIRT6 (n = 18, 9.05 years, 95% C.I. 5.59-12.5) when compared to those whose tumours express high levels of SIRT6 (n = 17, 12.14 years, 95% C.I. 9.67 -14.6, Kaplan-Meier p = 0.067).

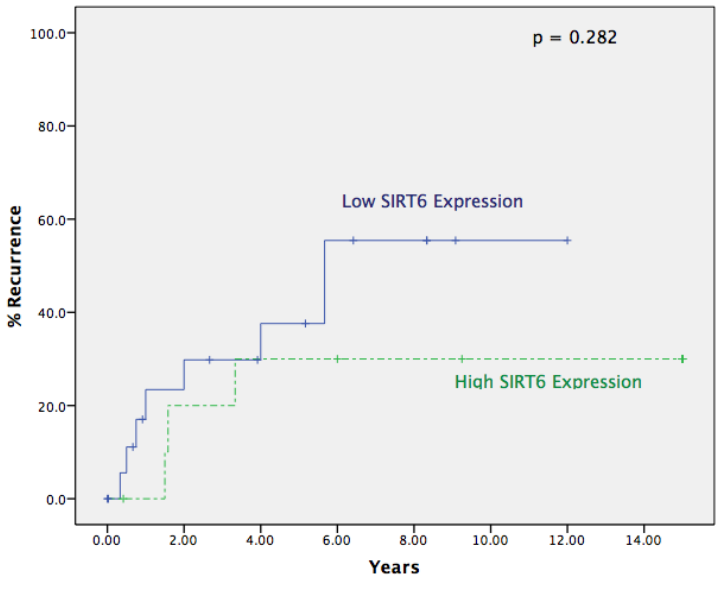

Figure 3.84: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT6 expression in patients with ER^{+ve} tumours.

High and low SIRT6 expression groups were determined as above or below the upper tertile cutoff value. The recurrence plot showed that time to recurrence was significantly shorter in patients with tumours that expressed low levels of SIRT6 (n = 20, 6.95 years, 95% C.I. 4.48-9.42) when compared to those whose tumours express high levels of SIRT6 (n = 11, 11.14 years, 95% C.I. 7.47, 14.8, Kaplan-Meier p = 0.282).

3.6.6 Conclusion

This study investigated the relative transcriptional expression levels of the SIRT6 gene in (25) normal, (73) non-malignant and (70) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT6 as the data were not normally distributed in all patient groups.

The data for SIRT6 gene expression variation between biopsies showed that there were significant differences in SIRT6 gene expression between biopsies from malignant, non-malignant and normal patient groups. The expression level of SIRT6 was significantly lower in malignant compared to both normal and non-malignant patient groups. SIRT6 gene expression increased significantly in the non-malignant when compared to normal patient groups.

The relative transcriptional expression level of SIRT6 showed significant association only with tumour grade, when breast cancer patients were divided according to the known histopathological markers. There was a significant correlation between decreased SIRT6 gene expression and higher tumour grade.

The Kaplan-Meier analysis for cancer specific survival according to relative transcriptional expression levels of SIRT6 showed that there was a significant association between SIRT6 levels and survival period in all patients cohort and in patients' subgroups, selected to have moderate prognosis (ER^{+ve} and NPI between 3.4 and 5.4). Breast cancer patients with tumours that expressed low levels of SIRT6, were 1.5 times more likely to die as a consequence of the breast cancer disease, than those with tumours expressing high levels of SIRT6.

Multivariate Cox-regression analysis demonstrated that the relative transcriptional level of the SIRT6 gene was independent of tumour size, grade, nodal status, oestrogen receptor status, lymphovascular invasion status, and the NPI in influencing survival.

The Kaplan-Meier analysis for recurrence time according to relative transcriptional expression levels of SIRT6 showed that there was a trend towards a significant difference between decreased levels of SIRT6 and shorter recurrence time, only in breast cancer patients selected to have a moderate prognosis according the NPI.

3.7 The relative transcriptional expression levels of SIRT7 in breast biopsies.

3.7.1 The relative transcriptional expression levels of SIRT7 and patients age.

The relative transcriptional expression levels of SIRT7 gene were determined in 25 normal biopsies, (76) non-malignant and (72) malignant (Table 3.23).

Table 3.23 Patient biopsies used in the SIRT7 study.

Sample Type		Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Relative SIRT7 Expression	Normal	25	96.20%	1	3.80%	26	100.00%
	Non-Malignant	76	97.40%	2	2.60%	78	100.00%
	Malignant	72	98.60%	1	1.40%	73	100.00%

The correlation between the level of SIRT7 relative transcriptional expression and patient's age was investigated in a patient group that combines both normal and non-malignant patients (Figure 3. 85). The median age of this patient group was 51years (20years - 84years). This data showed that there was negative correlation between the relative transcription levels of SIRT7 and chronological age. However, this correlation was not significant (Spearman's rank correlation coefficient; p = 0.119; Figure 3.85 b).

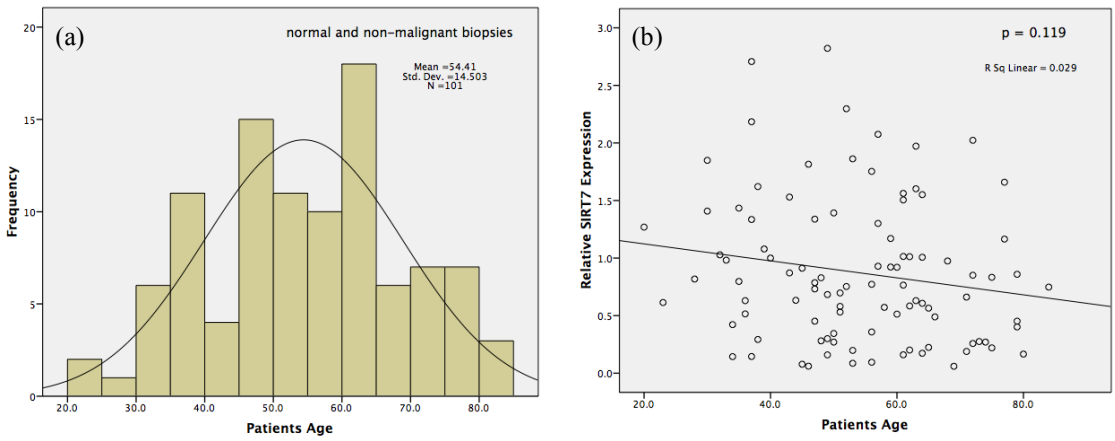

Figure 3.85: (a) Distribution of patient's age in patient group that combines both normal and non-malignant patients. (b) Correlation between relative transcription levels of SIRT7 and patient's age in patient group that combines both normal and non-malignant patients (Spearman test, p = 0.173).

3.7.2 Data distribution of the relative transcriptional expression levels of SIRT7.

The distribution of the relative transcriptional gene expression of SIRT7 was investigated in the three different studied biopsy types, (Figure 3.86). The histograms of the distribution of the relative transcriptional gene expression of SIRT7 showed that the range of the SIRT1 gene expression values was not normally distributed; instead the SIRT7 gene expression distribution was positive skewed in all groups (Figure 3.86). Therefore, non-parametric statistical tests were used for future data analysis for the relative transcriptional gene expression of SIRT7.

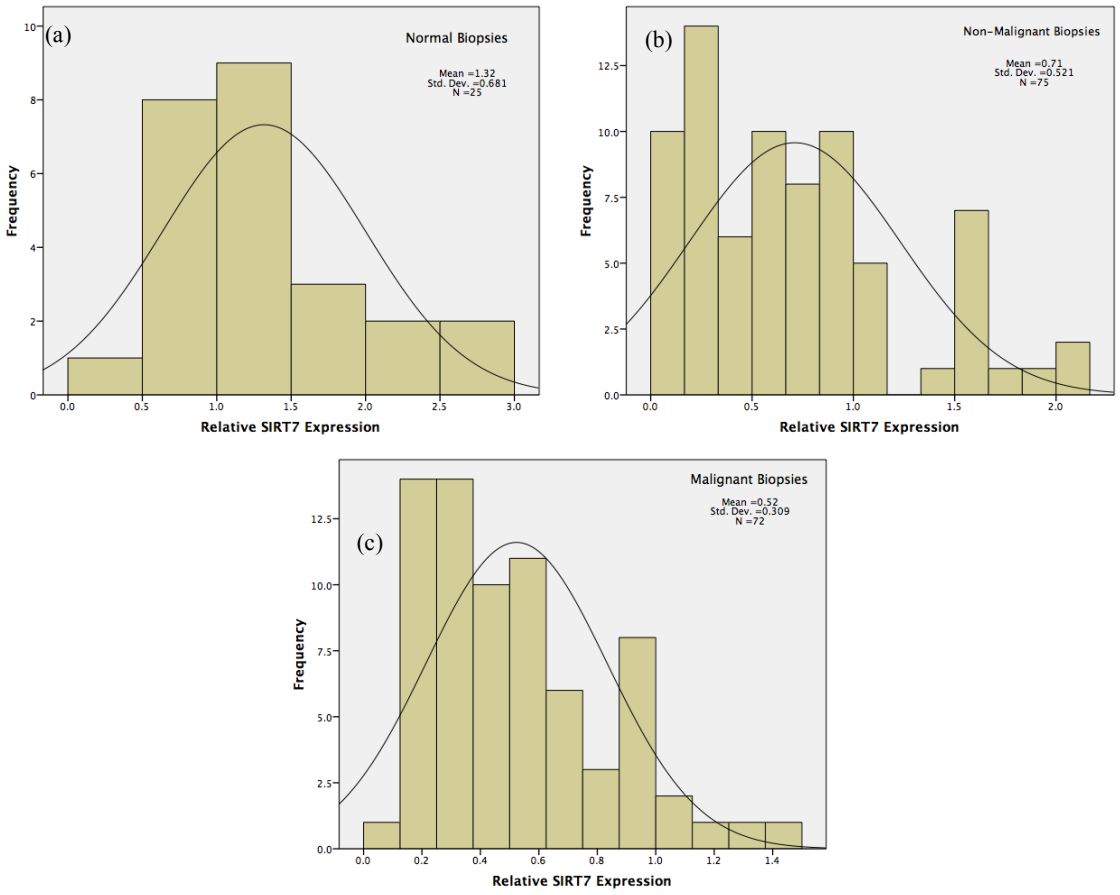

Figure 3.86: Distribution of the relative transcriptional gene expression of SIRT7 in three different groups.

(a) Relative transcriptional gene expression of SIRT7 in normal breast biopsies. (b) Relative transcriptional gene expression of SIRT7 in non-malignant breast biopsies. (c) Relative transcriptional gene expression of SIRT7 in malignant breast biopsies.

3.7.3 Comparison of the relative transcriptional expression levels of SIRT7 between patient groups.

The level of SIRT7 relative transcriptional expression was compared between all three groups. A Kruskal-Wallis test demonstrated that the relative transcriptional expression level for SIRT7 was significantly different between the three groups of breast biopsies ($p < 0.001$, Figure 3. 87). This experiment showed that normal samples have significantly higher levels of SIRT7 gene expression compared to both non-malignant and malignant samples ($p < 0.001$, Figure 3.87).

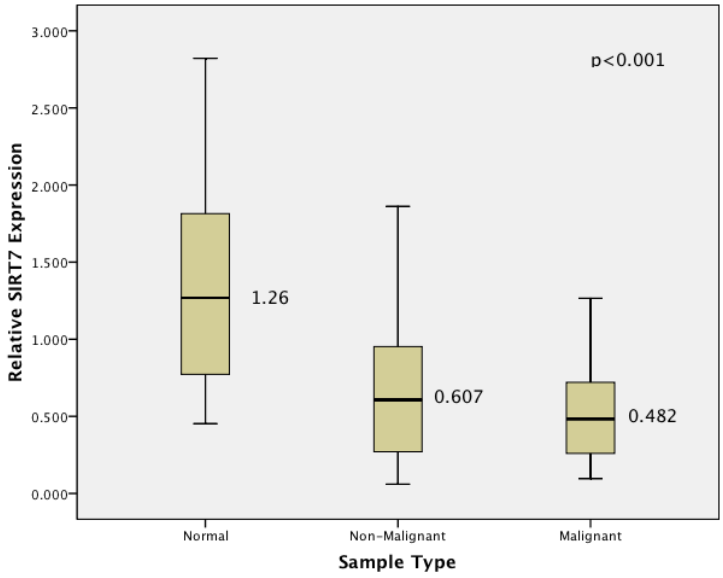

Figure 3.87: Box plot showing the transcriptional expression of SIRT7 in normal, non-malignant and malignant breast biopsies, relative to HPRT.

($p < 0.001$). The median value for each patient group is shown in the clear box to the right of each box plot.

Pairwise comparisons of the three groups using the Mann-Whitney test showed that the normal groups differed significantly (Table 3.6) in SIRT7 gene expression from both non-malignant ($p < 0.001$) and malignant ($p < 0.001$) groups. However, there was no significant difference in the levels of SIRT7 gene expression between the non-malignant and malignant groups ($p = 0.51$)

3.7.4 Comparison of the relative transcriptional expression levels of SIRT7 according to histopathological markers.

The relationships between SIRT7 and the standard histopathological parameters commonly used in describing breast cancer tumours were investigated and analysed. The data for each parameter is presented in the following sections.

3.7.4.1 Comparison the relative transcriptional expression levels of SIRT7 according to tumour grades.

Analysis of the data for the relationship between SIRT7 gene expression and tumour grade showed a trend toward significance at the 5% significance level when all three tumour grades were compared together (Kruskal-Wallis test $p < 0.067$) (Figure 3. 88). Assuming a valid trend then the level of SIRT7 expression decreases with increasing severity (higher grade) of tumour.

Pairwise comparisons of the three groups using the Mann-Whitney test showed that there was a marginal significant increase (Table 3.6) in the levels of SIRT7 in G 1 tumours when compared to both G2 and G3 ($p = 0.048$ and $p = 0.062$ respectively, Figure 3. 88).

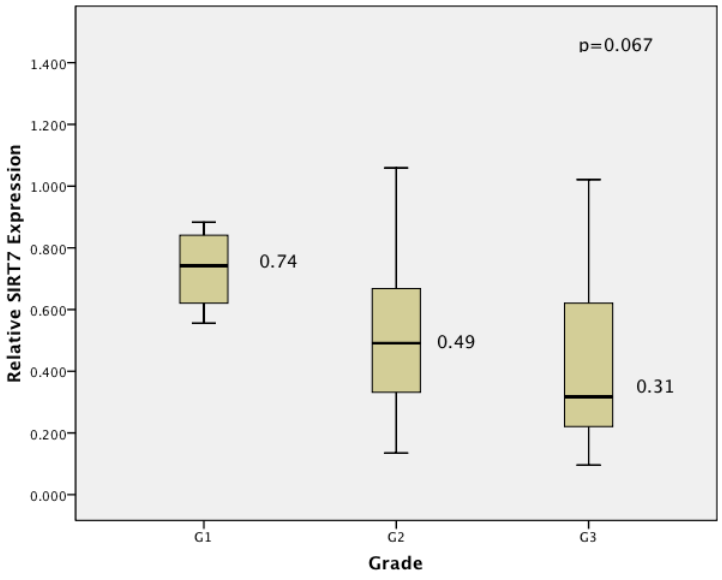

Figure 3.88: Box plot showing the relative transcriptional expression of in breast cancer biopsies by tumour grade.

n = 4 for grade 1, n = 32 for grade 2 and n = 29 for grade 3 *(*Kruskal Wallis Test, *p = 0.002)*. The median value for each patient group is shown in the clear box to the right of each box plot.

3.7.4.2 Comparison of the relative transcriptional expression levels of SIRT7 according to tumour size, nodal status, oestrogen receptor status and lymphovascular invasion.

Analysis of the data for the relationship between SIRT7 gene expression and tumour size, nodal status, ER status and lymphovascular invasion all showed no significant difference in the relative transcriptional expression levels of SIRT7 between any categories when compared together (Figure 3. 89 a, b, c and d). Table 3.8 shows the Kruskal-Wallis test p values for all comparisons.

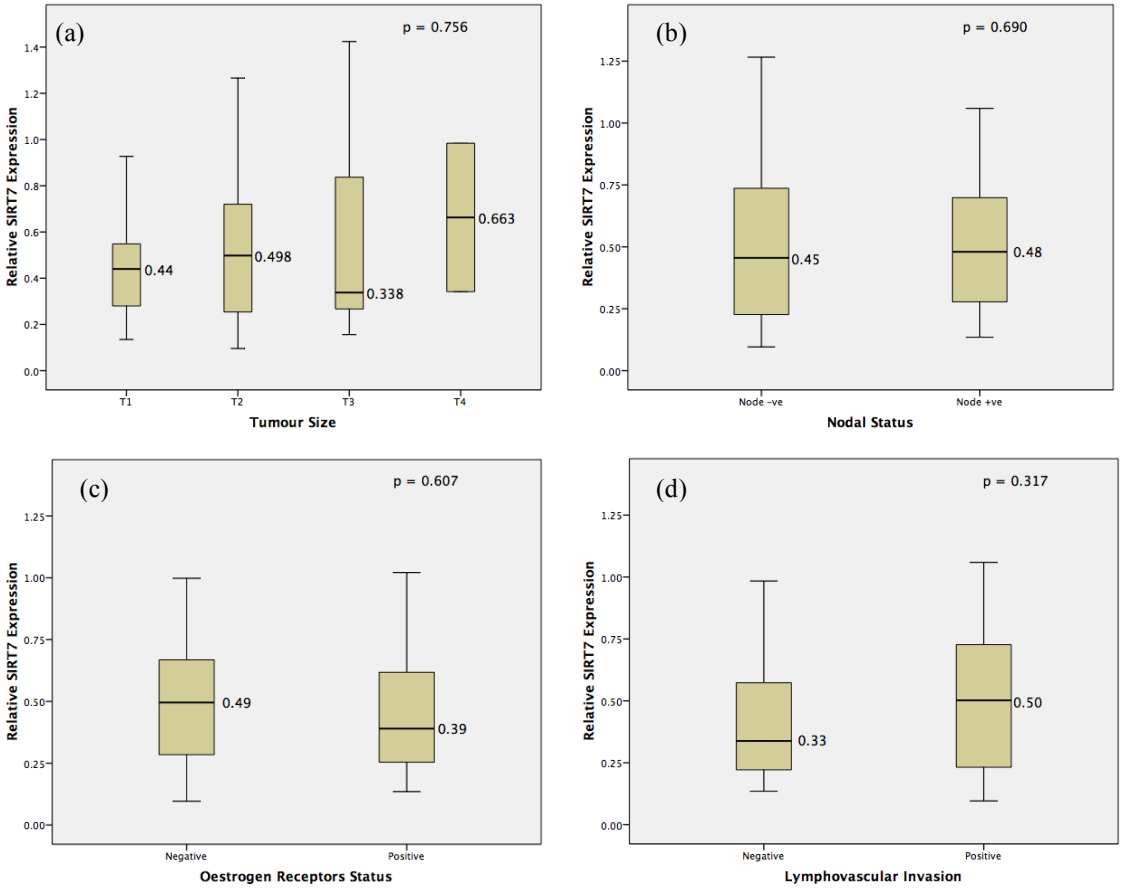

Figure 3.89: Box plots showing the relative transcriptional expression of SIRT7 in breast cancer biopsies by known prognostic markers:

a) tumour size, n = 11 for T1, n = 45 for T2 , n = 13 for T3 and n = 2 for T4 *(*Kruskal Wallis Test, *p = 0.756).* b) nodal status n = 20 for node negative and n = 43 for node positive, *(*Kruskal Wallis Test p = 0.690), c) oestrogen receptor status n = 20 for ER negative and n = 32 for ER positive (Kruskal Wallis Test, p = 0.607*). d) lymphovascular invasion, n = 19 for negative lymphovascular invasion and n = 29 for positive negative lymphovascular invasion (Kruskal Wallis Test, p = 0.317*). The median value for each patient group is shown in the clear box to the right of each box plot.

3.7.5 The relationship of the relative transcriptional expression levels of SIRT7 in breast cancer biopsies to patient survival and tumour recurrence.

In order to investigate the relationship between patient survival, tumour recurrence and SIRT7 relative gene expression, the patients were grouped into two groups using the median level of SIRT7 gene expression as a cutoff value. Table 3.24 and Table 3.26 show the numbers of patients included in each group.

3.7.5.1 SIRT7 and survival

3.7.5.1.1 Survival analysis in entire patient cohort according to SIRT7 levels.

Analysis of the survival data for the entire patient group (Table 3.24) using the Kaplan-Meier test showed that the survival period was significantly shorter in patients with tumours that expressed low levels of SIRT7 (n = 35, 9.82 years, 95% C.I. 7.74 -11.90) when compared to those whose tumours expressed high levels of SIRT7 (n = 34, 12.64 years, 95% C.I. 11.10-14.17, Kaplan-Meier p = 0.05, Figure 3.90).

Table 3.24: Number of breast cancer patient and cancer specific deaths of both patient groups included in the Kaplan-Meier survival analysis.

SIRT7 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths	Patients Number	Number of Cancer specific deaths
Low SIRT7 gene expression	35	13	17	6	21	9
High SIRT7 gene expression	34	7	18	3	10	1
Overall	69	20	35	9	31	10

Multivariate Cox-regression analysis demonstrated that the relative transcriptional level of SIRT7 gene was not independent of tumour size, grade, nodal status, ER status, lymphovascular invasion, and NPI in influencing survival (p = 0.107, Table 3.25).

Table 3.25: Multivariate Cox-regression stepwise analysis for breast cancer specific survival for SIRT7 and prognostic factors.

P values, Hazard ratio (HR) for death and confidence interval (CI) for the HR for SIRT7 and each prognostic factors are displayed in the table.

		Sig.	HR	95.0% CI Lower	Upper
Step 1	SIRT7	0.1	1.612	0.735	34.19
	Tumour Grade	0.503	-0.65	0.078	3.499
	Tumour Size	0.005	-3.228	0.004	0.386
	N	0.054	-2.754	0.004	1.052
	ER	0.126	1.336	0.686	21.104
	LV	0.623	-0.423	0.121	3.544
	NPI	0.759	0.376	0.132	16.026
Step 2	SIRT7	0.098	1.505	0.757	26.795
	Tumour Grade	0.525	-0.452	0.157	2.57
	Tumour Size	0.004	-3.108	0.005	0.364
	N	0.011	-2.432	0.014	0.572
	ER	0.129	1.291	0.687	19.251
	LV	0.651	-0.386	0.128	3.609
Step 3	SIRT7	0.106	1.448	0.736	24.614
	Tumour Grade	0.6	-0.363	0.18	2.696
	Tumour Size	0.003	-2.981	0.007	0.364
	N	0.011	-2.402	0.014	0.572
	ER	0.071	1.436	0.886	19.959
Step 4	SIRT7	0.107	1.373	0.744	20.964
	Tumour Size	0.002	-3.034	0.007	0.338
	N	0.01	-2.443	0.014	0.555
	ER	0.059	1.491	0.945	20.874

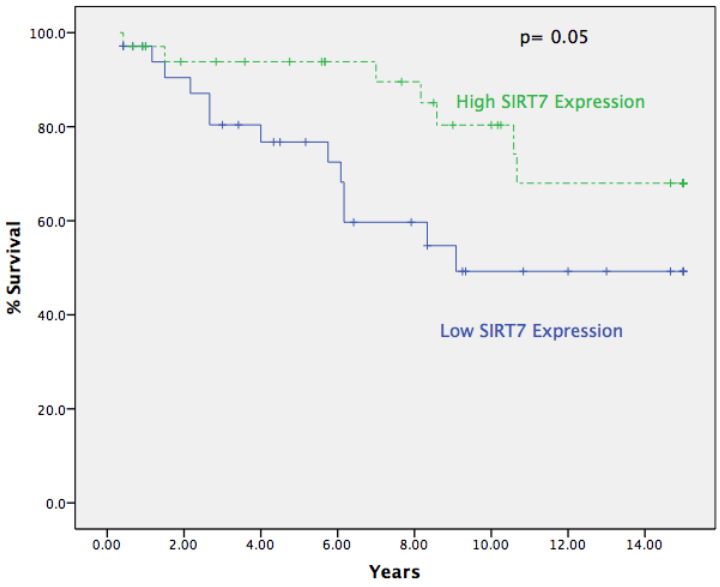

Figure 3.90: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT7 expression.

High and low SIRT7 expression groups were determined as above or below the median cutoff value. The survival curve shows a significant decrease in the survival period for patients with tumours that express low levels of SIRT7 (n = 35, 9.82 years, 95% C.I. 7.74 - 11.90) when compared to those whose tumours express high levels of SIRT7 (n = 34, 12.64 years, 95% C.I. 11.10-14.17, Kaplan-Meier p = 0.05).

3.7.5.1.2 Survival analysis in NPI selected patients according to SIRT7 levels.

Analysis of the survival data in patients with moderately good prognosis (NPI between 3.4-5.4, Table 3. 24) demonstrated that there was no significant difference in the cancer specific survival period in patients with tumours that expressed low levels of SIRT7 (n = 17, 10 .94 years, 95% C.I. 8.46-13.42) when compared to those whose tumours expressed high levels of SIRT7 (n = 18, 13.42 years, 95% C.I. 11.74 -15.10, Kaplan-Meier p = 0.1, Figure 3.91).

3.7.5.1.3 Survival analysis in ER^{+ve} patients according to SIRT7 levels.

Analysis of the survival data for the ER^{+ve} patient group (Table 3.24) using the Kaplan-Meier test showed a significant decrease in the survival period in patients with tumours that expressed low levels of SIRT7 (n = 21, 8.80 years, 95% C.I. 6.12-11.48) when compared to those whose tumours expressed high levels of SIRT7 (n = 10, 13.86 years, 95% C.I. 11.82 –15.89, Kaplan-Meier p = 0.045, Figure 3.92).

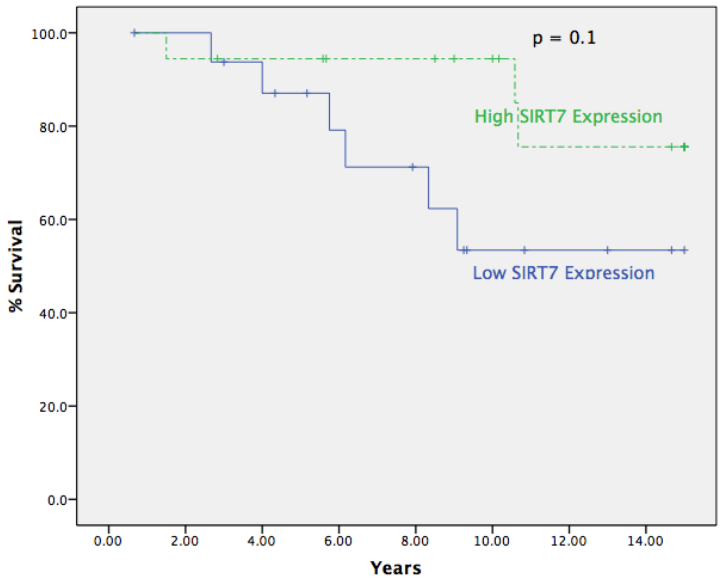

Figure 3.91: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT7 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

H High and low SIRT7 expression groups were determined as above or below the median cutoff value. The survival curve shows a non-significant decrease in the survival period for patients with tumours that express low levels of SIRT7 (n = 17, 10 .94 years, 95% C.I. 8.46-13.42) when compared to those whose tumours express high levels of SIRT7 (n = 18, 13.42 years, 95% C.I. 11.74 -15.10, Kaplan-Meier p = 0.1).

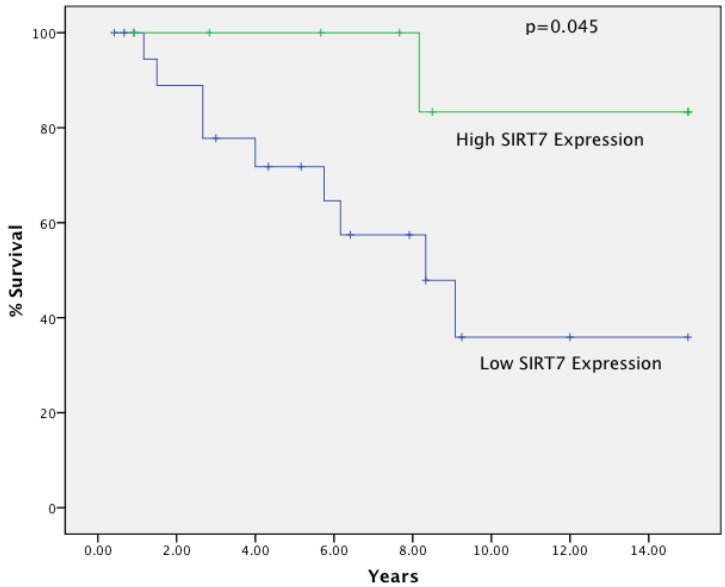

Figure 3.92: Kaplan-Meier Survival plot for breast cancer patients according to the level of SIRT7 expression in patients with ER^{+ve} tumours.

High and low SIRT7 expression groups were determined as above or below the median cutoff value. The survival curve shows a significant decrease in the survival period in patients with tumours that express low levels of SIRT7 (n = 21, 8.80 years, 95% C.I. 6.12-11.48) when compared to those whose tumours express high levels of SIRT7 (n = 10, 13.86 years, 95% C.I. 11.82 –15.89 , Kaplan-Meier p = 0.045).

3.7.5.2 SIRT7 and recurrence.

3.7.5.2.1 Recurrence analysis in entire patient cohort according to SIRT7 levels.

Recurrence of tumours was analysed using the Kaplan-Meier test, in all breast cancer patients, patient numbers are shown in Table 3. 26. There was no significant differences in the recurrence interval between patients with tumours that expressed low levels of SIRT7 (n = 34, 11.45 years, 95% C.I. 9.22-13.68) when compared to those whose tumours expressed high levels of SIRT7 (n = 35, 11.14 years, 95% C.I. 9.03 -13.25, Kaplan-Meier p = 0.677, Figure 3.93).

Table 3.26: Number of breast cancer patient and tumour recurrences of all breast cancer patient groups included in the Kaplan-Meier survival analysis.

SIRT7 Tertile	All breast cancer patients		Breast cancer patients with NPI between 3.4-5.4		Breast cancer patients with ER^{+ve}	
	Patients Number	Number of recurrences	Patients Number	Number of recurrences	Patients Number	Number of recurrences
Low SIRT7 gene expression	34	7	17	5	21	6
High SIRT7 gene expression	35	11	19	7	11	5
Overall	69	18	36	12	32	11

3.7.5.2.2 Recurrence analysis in NPI selected patients according to SIRT7 levels.

When tumour recurrence among the NPI-selected patient group was analysed (Table 3. 26), there was no significant difference according to the level of SIRT7 gene expression in patients with tumours that expressed low levels of SIRT7 (n = 17, 10.67 years, 95% C.I. 7.67-13.68) when compared to those whose tumours expressed high levels of *SIRT7* (n = 19, 10.86 years, 95% C.I. 7.86 -13.85, Kaplan-Meier p = 0.994, Figure 3.94).

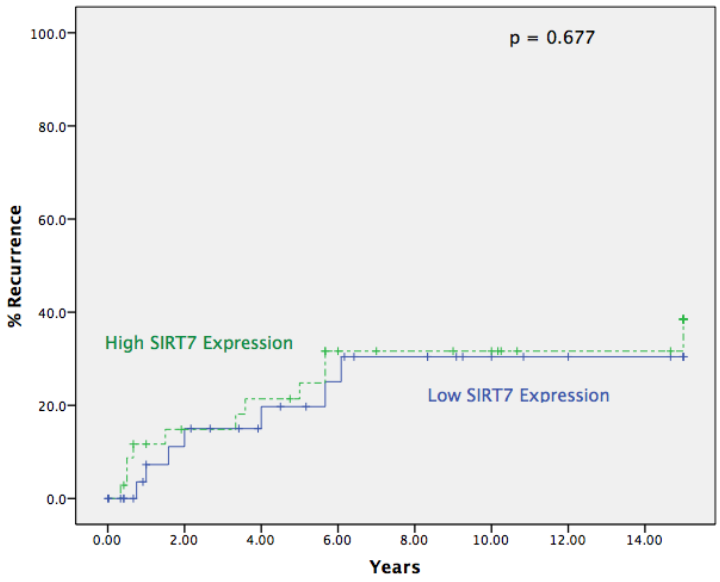

Figure 3.93: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT7 expression.

High and low SIRT7 expression groups were determined as above or below the median cutoff value. The recurrence curve showed a non significant difference in the time to recurrence in patients with tumours low levels of SIRT7 (n = 34, 11.45 years, 95% C.I. 9.22-13.68) when compared to those whose tumours express high levels of SIRT7 (n = 35, 11.14 years, 95% C.I. 9.03 -13.25, Kaplan-Meier p = 0.677).

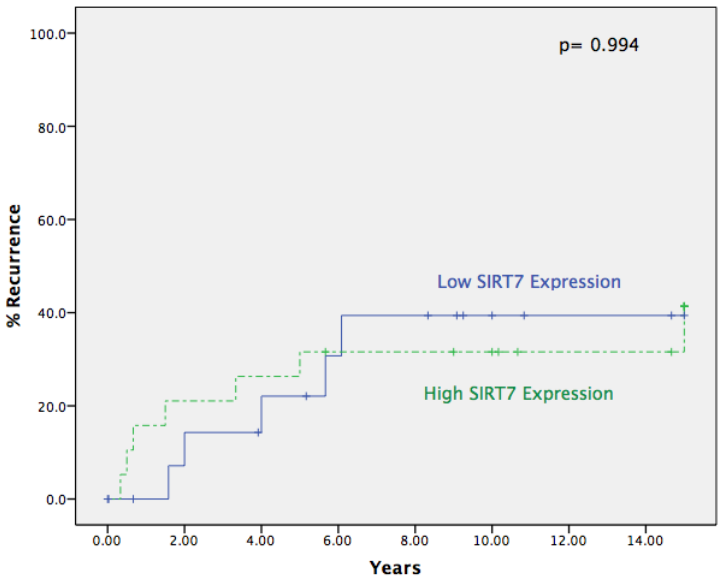

Figure 3.94: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT7 expression in patients group with moderate prognosis (NPI between 3.4 and 5.4).

High and low SIRT7 expression groups were determined as above or below the median cutoff value. The recurrence curve showed a non-significant difference in the time to recurrence period in patients with tumours that express low levels of SIRT7 (n = 17, 10.67 years, 95% C.I. 7.67-13.68) when compared to those whose tumours express high levels of SIRT7 (n = 19, 10.86 years, 95% C.I. 7.86 -13.85, Kaplan-Meier p = 0.994).

3.7.5.2.3 Recurrence analysis in ER^{+ve} patients according to SIRT7 levels.

Further analysis of recurrence within the subgroup of patients with ER^{+ve} tumours (Table 3.26) failed to reveal any significant difference in time to recurrence between patients with tumours that expressed low levels of SIRT7 (n = 21, 10 years, 95% C.I. 6.86 -13.14) when compared to those whose tumours expressed high levels of SIRT7 (n = 13, 9.21 years, 95% C.I. 5.38-13.04, Kaplan-Meier p = 0.684, Figure 3.95)

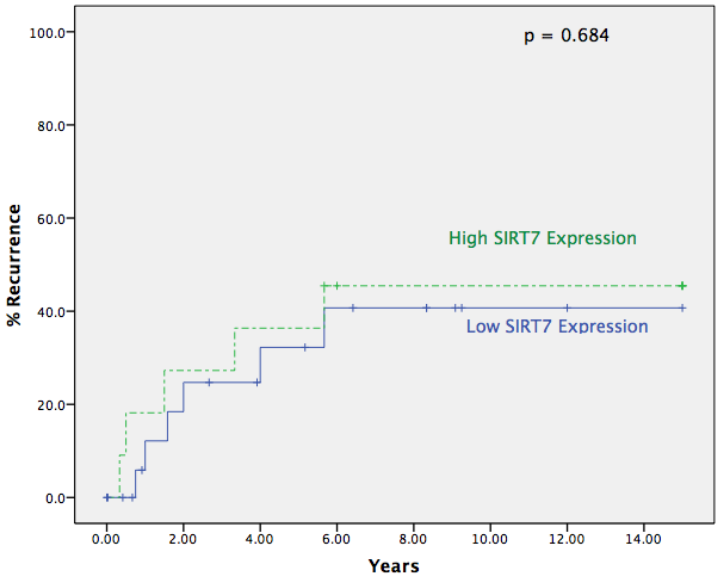

Figure 3.95: Kaplan-Meier recurrence plot for breast cancer patients according to the level of SIRT7 expression in patients with ER^{+ve} tumours.

High and low SIRT7 expression groups were determined as above or below the upper median value. The recurrence curve showed a non-significant difference in the time to recurrence in patients with tumours that express low levels of SIRT7 (n = 21, 10years, 95% C.I. 6.86 -13.14) when compared to those whose tumours express high levels of SIRT7 (n = 13, 9.21 years, 95% C.I. 5.38-13.04, Kaplan-Meier p = 0.684).

3.7.6 Conclusion

This study investigated the relative transcriptional expression levels of the SIRT7 gene in (25) normal, (76) non-malignant and (72) malignant breast biopsies. Non-parametric statistical tests were used to analyse the data for relative transcriptional gene expression of SIRT7 as the data distributions were positively skewed in all patient groups.

The data for SIRT7 gene expression variation between breast cancer biopsies showed that there were significant differences in SIRT7 gene expression between biopsies from malignant, non-malignant and normal patient groups. The expression level of SIRT7 was significantly higher in normal compared to both non-malignant and malignant patient groups. There was no significant difference in the levels of SIRT7 expression between non- malignant and malignant patient groups.

There was a marginal significant increase in the levels of SIRT7 in G1 tumours when compared to both G2 and G3 ($p = 0.048$ and $p = 0.062$ respectively).

The Kaplan-Meier analysis for cancer specific survival according to relative transcriptional expression levels of SIRT7 showed that there was a significant association between higher levels of SIRT7 and longer survival period in all patients cohort and in ER^{+ve} breast cancer patients. However, SIRT7 gene expression was not independent of tumour size, grade, nodal status, ER status, lymphovascular invasion, and NPI in influencing survival.

Chapter IV

Discussion 1

The relative transcriptional expression of sirtuins in breast cancer

4.1 Summary of experimental results.

The relative transcriptional expression levels of the sirtuin genes were investigated in (25) normal, (78) non-malignant and (73) malignant breast biopsies. Non-parametric tests were used to compare the relative transcriptional expression levels of the Sirtuin genes between these three groups and to compare sirtuins levels in breast cancer biopsies grouped according to the known histopathological and prognostic factors. The Kaplan-Meier statistical analysis was used to investigate whether there was significant association between the relative transcriptional expression levels of the sirtuin genes, tumour recurrence and cancer specific survival.

This discussion is based on the significant results of this experiment that are presented in Table 6.1: refer to Table 3.6 for p values.

Table 4.1:summary of the significant experimental results for sirtuins genes expression in breast biopsy.

Genes	Significant results
The relative transcriptional expression of SIRT1 gene	Lower in malignant when compared with normal and non-malignant breast biopsies. Association between decreased levels and higher tumour grade. Lower levels are associated with longer survival period (patient selected according to NPI between 3.4-5.4).
The relative transcriptional expression of SIRT2 gene	Lower in malignant when compared with normal and non-malignant breast biopsies. Association between decreased levels and higher tumour grade.
The relative transcriptional expression of SIRT3 gene	Lower in malignant when compared with normal and non-malignant breast biopsies. Association between decreased levels and higher tumour grade.
The relative transcriptional expression of SIRT4 gene	Lower in malignant when compared with non-malignant breast biopsies. Positive correlation with increasing age. Lower levels are associated with early recrrence in ER^{+ve} breast cancer patients.
The relative transcriptional expression of SIRT5 gene	Lower in normal when compared with non-malignant and malignant breast biopsies. Trend of decreased expression associated with malignant changes when compared to non-malignant.
The relative transcriptional expression of SIRT6 gene	Lower in malignant when compared with normal and non-malignant breast biopsies. Association between decreased levels and higher tumour grade. Higher levels are associated with longer survival period in all patients cohort and in patient selected with (ER^{+ve} and NPI between 3.4 and 5.4). Independent predictor of survival.
The relative transcriptional expression of SIRT7 gene	Higher in normal when compared with non-malignant and malignant breast biopsies. Higher in grade 1 when compared with grade 2 Higher levels are associated with longer survival period in all patients cohort and in ER+ve breast cancer patients.

The work presented in this thesis differs technically in two key ways from the study by (Ashraf *et al*, 2006). The number of patients has been increased, a new control group added, and HPRT used as the housekeeping gene. Crucially, the validation experiments reported here in relation to the use of 18S rRNA demonstrate that 18S rRNA was not ideal as a housekeeping gene comparator for any of these genes, and very poor for SIRT4, SIRT5, and SIRT6 (Chapter 3.1). Therefore, HPRT was used instead of the 18S rRNA gene as a housekeeping gene after being validated. A review of the validation experiment for the previous study by (Ashraf *et al*, 2006) supported this decision (Appendix 3). One explanation for these observations is that the combination of increased patient numbers and a more appropriate housekeeping gene comparator, may contribute to the observed differences between the two studies and indicates that the work presented here should be regarded as more appropriate.

4.2 Breast biopsies and the characteristics of patient cohorts.

Patient's age was significantly different in normal when compared with patient's age in both non-malignant and malignant groups. Since, the incidence of breast cancer increases rapidly with age, it was expected that average age of patients with breast cancer disease (61 years would be greater than those (young ladies) who require reduction mammoplasty (41.6).

This study confirms that the presence, or absence of axillary nodal metastases is the single most important prognostic factor, for both survival and recurrence in breast cancer. The loss of significance for other prognostic markers in this patient cohort is likely to be due to the small number and the unequal distribution of cancer biopsies according to these prognostic factors. The NPI was able to correct for these differences and showed significant association with cancer specific survival in this study.

4.3 The relative transcriptional expression level of Sirtuin in breast biopsies.

4.3.1 The relative transcriptional expression level of SIRT1 in breast biopsies.

The decreased levels of SIRT1 with increasing age in the "normal patient group" is consistent with another study showing that the level of SIRT1 decreases significantly with serial cell passage in both human and mice cells (309).

The anti-apoptotic and pro-apoptotic effects of SIRT1 reflect its complicated biological functions, and are indicative of its intricate potential involvement in cancer disease (refer to introduction chapter; 1.6.2). The data presented here is interesting, as it is consistent with the anti-tumourgenic effect of SIRT1. Several studies suggest that SIRT1 may act as a tumour suppressor. MEFs derived from SIRT1-null mice are prone to spontaneous immortalization, suggesting that SIRT1 behaves as a growth-suppressive gene in culture (310). Furthermore, hematopoietic stem cells from SIRT1-null mice have increased proliferation potential, and siRNA knockdown of SIRT1 in human fibroblasts accelerates cell proliferation (230, 326). SIRT1 has also been shown to inhibit androgen receptor

dependent cell proliferation in prostate tumour cells (277). Furthermore, transgenic over-expression of SIRT1 in the intestine, inhibited polyp formation in the ApcMin mice (327), whereas SIRT1 deficiency led to increased tumour formation in p53-null mice (328). These observations suggest that SIRT1 may suppress tumour growth under certain conditions, and that SIRT1 activators could be used for cancer treatment, or prevention (329).

The decreased relative transcriptional expression levels of SIRT1 observed in this study, in breast cancer biopsies, when compared with both normal and non-malignant biopsies, is consistent with a recent study reporting increased levels of SIRT1 in normal colon mucosa and benign adenomas, and decreased levels of SIRT1 in approximately 30% of carcinomas (330). Consistent with this effect, it has been found that elimination of SIRT1 accelerates tumour xenograft formation by HCT116 cells, whereas SIRT1 over expression inhibits tumour formation (330). Furthermore, the pharmacological inhibition of SIRT1 stimulates cell proliferation under conditions of growth factor deprivation (330).

This study also showed that decreased relative transcriptional levels of SIRT1 gene were associated with increasing tumour grade (Figure 3.20). Our finding is in consistent with the study by Kabra et al showing that over-expression of SIRT1 was observed in 25% of stage I/II/III colorectal adenocarcinomas, but rarely found in advanced stage IV tumours (330).

Kabra et al 2009 suggested that hyper-phosphorylation of pRb as an indirect result of SIRT1 inhibition, might be responsible for this tumourigenic effect of decreased SIRT1. A decrease in SIRT1 activity results in activation of E2F1 thus promoting pRb hyper-phosphorylation indirectly through inducing cyclinD/cdk4 phosphorylation activity (330).

Furthermore, decreased relative transcriptional expression of SIRT1, as observed in breast malignant biopsies, might promote tumourogenesis through the known interaction between SIRT1 and NFκB. SIRT1 can deacetylate and inactivate NF-κB, thereby sensitising cells to tumour necrosis factor α (TNFα) induced apoptosis (218). Therefore a decreased level of SIRT1 might be beneficial to the tumour cell because it will increase NF-κB activity, thus promoting cell proliferation. Furthermore, it has been reported that in breast cancer, SIRT1 is recruited to suppress the activity of NF-κB by neddylated breast cancer associated protein 3 (BCA-3) (261). This provides an alternative way in which decreased SIRT1 activity can enhance the transcriptional activity of NF-κB

The anti-cancer effect of SIRT1 observed in this study is probably due to the interaction between SIRT1 and NFκB, an important regulator of ageing-related cellular processes. This result might indicate a potential role for NF-kB in breast cancer tumourigenesis that might be promoted thorough decreased levels of SIRT1.

This study, along with that of Kabra et al, provides evidence that decreased SIRT1 at both mRNA and protein level is associated with cancer (breast and colon). This study showed that decreased relative transcriptional expression levels of SIRT1 were associated with malignant breast biopsies and with increasing tumour grade (anti-cancer effect).

It is therefore surprising that high levels of SIRT1 are shown to be associated with worse prognosis in breast cancer disease (cancer promoting effect). The data presented here showed that higher levels of the relative transcriptional expression of SIRT1, rather than lower levels are associated with worse survival and early recurrence in patients with moderate prognosis according to the NPI. These data seem to be consistent with a cancer promoting effect of SIRT1.

There are two possible hypothesises to explain our findings:

There is an apparent contradiction between the observed results for SIRT1; between results indicating that increased levels of SIRT1 are associated with poor prognosis in breast cancer patients and the results showing decreased levels of SIRT1 are associated with higher tumour grade. Possible explanations for this are either, that in tumours with poor prognosis, increased SIRT1 is an important primary component of tumourigenesis (primary event), or that the affected cells are attempting to enter apoptosis by increasing SIRT1 despite the ongoing tumourigenesis process (secondary response).

Primary event: Increased SIRT1 might cause different patterns (motifs) of TSGs expression, especially of those who have regulatory effect on SIRT1.

These tumour suppresser genes are associated with cancer disease. Therefore, changes in gene silencing profile, mutations, and/ or expression of any of the TSGs which are involved in regulating SIRT1 might be responsible for the increase in the relative transcriptional levels of SIRT1 that is associated with shorter survival and early recurrence in breast tumours that already showed low levels of SIRT1.

If SIRT1 suppresses certain TSGs, there may be a resulting increase in SIRT1 expression itself because of the loss of known TSG repression of SIRT1 transcription. This could also happen independently of any involvement of SIRT1 with TSGs, for example, in the case of a loss of function mutation in a TSG like p53. It has been shown that SIRT1 could be regulated by different tumour suppressor genes such as p53 and DBC1 (247, 250, 322, 344). SIRT1 can be regulated by p53 directly in a FOXO-dependent manner (247),or indirectly thorough trans-activating HIC1 transcription, a protein that binds directly to the SIRT1 promoter and represses its expression. (248). Furthermore, indirect control of SIRT1 by p53 could be exercised by DeltaNp63alpha that down-regulates SIRT1 (249). Another TSG (DBC1) was shown to interact with SIRT1 and repress SIRT1 deacetyaltion of p53, resulting in an increase in p53-dependent apoptotic responses (250).

Secondary response: Increasing SIRT1 levels in clinically aggressive tumours might indicate a last resort defensive cellular response, aimed at deactivating the (NFκB) cancer promoting pathway, thus sensitising cancerous cells to apoptosis. This might be consistent with increased necrotic tissue in aggressive tumours.

To conclude, it might be possible that such subtle irregularity, or an individual variation of SIRT1 levels, that might result from either/ or primary event or secondary response, are responsible for such aggressive clinical behaviour for breast cancer tumours that express higher level of SIRT1, when compared with tumours that express low levels of SIRT1, in malignant biopsies with overall decreased levels of SIRT1, when compared to normal biopsies.

The observed association between decreased levels of SIRT1 and higher tumour grade observed in our study is consistent with the study by Kabra et al., reporting that SIRT1 over-expression was observed in 25% of stage I/II/III colorectal adenocarcinomas but is rarely found in advanced stage IV tumours (330). By definition, high-grade tumours are poorly differentiated tumours (Table 1.4). In keeping with the role of SIRT1 in growth and differentiation, it is possible that altered levels of SIRT1 are implicated in the loss of differentiation observed in high-grade tumours. It is impossible at present to explain these results fully in the light of the limited information currently available concerning the involvement of SIRT1 in embryonic development, growth and differentiation. However, it seems reasonable to suggest a potential role for SIRT1 in tumour differentiation state and histopathological appearance.

4.3.2 The relative transcriptional expression level of SIRT2 in breast biopsies.

SIRT2 might be implicated in cancer disease through its role in mitosis and cell cycle control. SIRT2 deacetylates the α-tubulin subunit of microtubules in the spindle apparatus resulting in cellular arrest during cytokinesis and SIRT2 overexpression results in delay in cell cycle progression (201). Furthermore, SIRT2 is negatively regulated by CDC14B phosphatase, released in late M phase, allowing cell cycle progression (286).

Our data showed that the relative transcriptional level of the SIRT2 gene was significantly decreased in the malignant biopsies, when compared with both normal and non malignant breast biopsies. This finding is consistent with the anti-proliferation effect of SIRT2 and in agreement with a previous study that reported down-regulation of SIRT2 in gliomas, and showed that ectopic expression of SIRT2 suppresses the growth of glioma cell lines (336).

There was no significant difference in the relative transcriptional levels of SIRT2 between the normal and non-malignant groups. This might indicate a non-significant difference in breast cell proliferation rate between the two groups, or might result from a cell cycle phase dependent expression of SIRT2.

The pathological grading system for breast cancer is based on tubule formation (differentiation), nuclear pleomorphism and mitotic count. The association between decreased levels of SIRT2 and higher tumour grade observed in this study might be indicative of increased mitotic count (rate of cell division) for grade 3 tumours. Furthermore, similar to SIRT1, a role for SIRT2 in mammalian development was suggested through its interaction with the homeobox transcription factor 10 (Hoxa10) (289). These findings might be supportive of the potential involvement of SIRT2 in development and differentiation.

This study did not support the suggested potential role for SIRT2 in tumour invasion and metastasis, as it did not show any significant association between SIRT2 gene expression and patient survival or tumour recurrence.

The findings of this work suggest that a decrease in SIRT2 activity might assist DNA-damaged tumour cells in escaping cell cycle arrest during tumour initiation and progression. Furthermore, the decrease in SIRT2 levels might also be implicated in the poor differentiation and higher mitotic count associated with higher tumour grades.

4.3.3 The relative transcriptional expression level of SIRT3 in breast biopsies.

The data presented in this study are consistent with a pro-apoptotic role for SIRT3 (295). The decreased levels of SIRT3 in breast cancer biopsies and in higher grade tumours might be indicative of suppression of the mitochondrial apoptotic pathway, thus implicating SIRT3 in tumourigenesis.

This study showed no significant difference in the relative transcriptional levels of SIRT3 between normal and non-malignant groups. In the light of the available data this could be interpreted by the absence of any significant difference in breast cell proliferation and/or metabolic status between the two groups.

4.3.4 The relative transcriptional expression level of SIRT4 in breast biopsies.

SIRT4 is one of the mitochondrial sirtuins and has a NAD-dependent ADP-ribosyltransferase activity. It down-regulates mitochondrial glutamate dehydrogenase within pancreatic β cells and it involvement in insulin/ glucose metabolism (204) represent its known biological function.

Therefore, it is impossible to interpret our results in the light of the available information concerning SIRT4. However, the observed decrease in SIRT4 levels in malignant, when compared with non-malignant breast biopsies and the significant association between recurrence and low levels of SIRT4 in ER^{+ve} breast cancer patients, is suggestive of potential involvement of SIRT4 in breast cancer disease and metastasis. The difference in levels of SIRT4 expression between non-malignant group and both normal and malignant breast biopsies might indicate changes in the metabolic status of cells resulting from, either increasing age, or changes in the available nutrients for normal breast tissue adjacent to malignant biopsies.

4.3.5 The relative transcriptional expression level of SIRT5 in breast biopsies.

Differences observed in this study in the relative transcriptional levels of SIRT5 between the three studied groups, is possibly due to changes in metabolic and or redox status, that are associated with ageing, or with cancerous transformation. These results cannot exclude, or confirm, the possible involvement of SIRT5 in the cancer disease process as the available information for SIRT5 is very limited.

4.3.6 The relative transcriptional expression level of SIRT6 in breast biopsies.

Our data are consistent with the anti-cancer effect of SIRT6. SIRT6 has a negative effect on cellular proliferation through its interaction with the NF-κB RELA subunit and deacetylation of histone at NF-κB target gene promoters. This interaction results in attenuating NF-κB signalling, increasing cellular sensitivity to stress-induced apoptosis (302). Our data demonstrate that the relative transcriptional expression of SIRT6 is significantly decreased in breast cancer biopsies, when compared with both normal and non-malignant tissue. Furthermore, low SIRT6 levels were associated with higher tumour grade, short survival period and early recurrence time.

Decreased SIRT6 expression in breast cancer biopsies could be indicative of increasing genomic instability associated with the development of cancer. Interestingly, this decrease in SIRT6 expression was also significantly associated with higher tumour grades. While the grading system takes into account nuclear pleomorphism (nuclear abnormalities in size, chromatin pattern and organization), which indicates the histological features of genomic and chromosomal abnormalities, SIRT6 expression may provide a molecular correlate of these histological features.

In mammals, regulation of genomic stability at the cellular level has been linked to both tumour suppression and ageing (345). Various pathways are employed to repair specific types of DNA damage. Double Strand Breaks (DSBs) are repaired by non-homologous end-joining or homologous recombination. Single Strand Breaks (SSBs) which result from endogenous oxidation, alkylation, and deamination events, are repaired mainly by BER. SIRT6 has been found to be functionally linked to BER and appears to function upstream of the polymerase β (Polβ) deoxyribosyl phosphate lyase reaction in BER (300). Polβ? is

the major polymerase used for base excision repair in mammals (346, 347). It has been suggested that SIRT6 may regulate BER indirectly, by creating accessibility for BER factors, via modification of histones or other chromatin related factors (300). Decreased expression of SIRT6 in breast cancer tissue is intriguing, as it may indicate an increased incidence of un-repaired and/or improperly repaired DNA damage. Such a situation would have serious consequences for the cell, potentially leading to cell death, senescence, de-regulation of cellular function, genomic instability, or oncogenic transformation.

Increased relative transcriptional expression of SIRT6 in non-malignant breast biopsies, when compared to normal breast tissue, may be indicative of the accumulation of age-associated DNA damage, as the normal group is significantly younger in age compared to the non-malignant group. Increased SIRT6 expression in the non-malignant group could be a compensatory mechanism to overcome the age-associated decreased activity of BER and polβ? (348, 349).

A number of other genes involved directly in DNA damage repair have been associated with breast cancer susceptibility. These include BRCA1 and BRCA2, which are responsible for 21%–40% of hereditary breast cancers that account for 5%–10% of all breast cancer cases (3, 4). BRCA1 plays an important role in the repair of DNA damage through associations with proteins involved in the repair of DNA double strand breaks (5, 6). Somatic BRCA1 mutations are observed rarely in sporadic breast cancer; however, both BRCA1 mRNA and protein expression are downregulated in approximately 30% of sporadic breast cancers and 70% of ovarian cancer cases (350). Characteristically, BRCA1-mutated breast tumours are poorly differentiated, occur at an early age of onset, are generally ER, progesterone receptor (PgR), epidermal growth factor receptor (HER2/neu) negative and are associated with a poor prognosis. The data obtained is in keeping with such a scenario, whereby the transcriptional expression of SIRT6 is decreased in breast cancer and lower SIRT6 expression is associated with grade 3 tumours and a shorter overall survival period. However, SIRT6 is involved in the repair of less severe DNA damage (SSBs) when compared with BRCA1 (DSBs). This could explain the older chronological age at which sporadic breast cancer occurs, when compared with familial breast cancers. Furthermore, these data suggest that decreased expression of SIRT6 might predispose individuals to sporadic breast cancer.

SIRT6 might be positively regulated by GCIP, a negative regulator of proliferation (106). GCIP is significantly down-regulated in several human tumours and may function as a tumour suppressor gene. Other than acting as a transcription repressor for cyclin D1, it is

possible that GCIP could suppress tumourigenesis and promote DNA damage resistance through its interaction with SIRT6 (106). Therefore, a decreased SIRT6 level, as observed in breast cancer, could be indicative of GCIP down regulation, thus promoting the disease.

The involvement of SIRT6 in inflammation has been reported recently (303). Although inflammation is not a risk factor in breast cancer, it might still be possible that changes in the inflammatory mediators, at the cellular level in mammary epithelial cells, are involved in breast cancer tumourigeneis. This possibility highlights the potential implication for SIRT6 in inflammation and cancer.

Our results show that SIRT6 is an independent predictor of survival, with a significant association between high levels of SIRT6 expression and a better prognosis. These data suggest that SIRT6 may be used as a biological marker for predicting survival outcome.

In addition, high levels of SIRT6 are significantly associated with longer survival in breast cancer patients with moderate prognosis (NPI between 3.4-5.4), or ER^{+ve} tumours. Moreover, there was a trend observed toward shorter recurrence time in patients with NPI between 3.4-5.4, who have tumours that express low levels of SIRT6.

This study has demonstrated a strong association between SIRT6 expression and breast cancer and suggests that SIRT6 might be implicated in breast cancer pathogenesis (initiation and progression). These findings indicate that the SIRT6 levels could be used as an additional prognostic marker in breast cancer patients, especially in those individuals who have equivocal prognostic pathological markers. It may be particularly useful in providing a more accurate prognosis for those breast cancer cases that behave unexpectedly, according to the known pathological prognostic markers. Therefore, stratifying breast cancer patients according to the level of SIRT6 expression might improve predicting breast cancer disease outcomes.

Furthermore, the association between high levels of SIRT6 and better tumour behaviour and disease prognosis indicates that enhancing SIRT6 activity may confer benefit to breast cancer patients. Our study suggests SIRT6 as potential novel anticancer agent.

4.3.7 The relative transcriptional expression levels of SIRT7 in breast biopsies.

The data presented in this study, showed that lower SIRT7 gene expression is prognostic of better long term patient survival and lower tumour recurrence in a patient group selected by NPI to have an intermediate clinical prognosis.

The involvement of SIRT7 in cancer disease is complicated, as the available evidence suggests both negative and positive effects on tumourigenesis associated with SIRT7. A recent study reported decreased expression of SIRT7 to be associated with different murine tumourigenic cell lines. This study only showed increased levels of SIRT7 in normal biopsies, when compared with both malignant and non-malignant breast biopsies, an effect that could be due to the age difference between the two groups. Nevertheless, our study does provide some support for a negative effect of SIRT7 on tumourigenesis, because of the significant association between decreased levels of SIRT7 and shorter survival period.

A potential mechanism for an anti-cancer action of SIRT7 is via activation of p53- and c-myc-dependent transcription, thus promoting apoptosis (306). Furthermore, decreased expression of SIRT7 was observed in murine tumourigenic cell lines: P19 (teratocarcinoma), NB41A3 (neuroblastoma), C3H/MCA (transformed fibroblast-derived cell line) as compared to the control non-tumourigenic cell line C3H/10T1/2 (306). Therefore, decreased SIRT7 levels have been suggested to promote the cancer disease process.

Chapter V

Sirtuin gene expression in response to anti-tumour drug treatment in breast cancer cell lines.

Introduction:

We have previously shown that sirtuin expression is altered in breast cancer and associated with survival. However, sirtuin expression has not been explored in the context of antitumour therapies. This study aimed to investigate sirtuin expression in different mammary cancer cell lines, in response to different antitumour treatments. Docetaxel and tamoxifen was chosen for this experiment as they represent the main two treatments commonly used in breast cancer patients. Tamoxifen is used in postmenopausal women with ER^{+ve} Tumour and Docetaxel is used in ER^{-ve} tumours.

5.1.1 Oestrogen receptor status of breast cancer cell lines.

Three different types of immortalized tumourigenic human mammary cell lines (MCF-7, MDA-MB-453 and MDA-MB-231) were used in this study, as described in the Materials and Methods. These cell lines represented both ER positive (MCF-7) and ER negative (MDA-MB-453 and MDA-MB-231) breast cancers. All three breast cancer cell lines showed epithelial characteristics when grown in culture media, as described in the literature (Figure 5.1). In the literature it was reported that different variants of the MCF-7 cell line are present and that MCF-7 cells may lose their ER+ve status with passage (351, 352). Altered ER status for the cell studied could change the response to tested drugs. Therefore, it was decided to test for ER status in all the studied breast cancer cell lines, using two different techniques (Immunofluorescence and Western blotting).

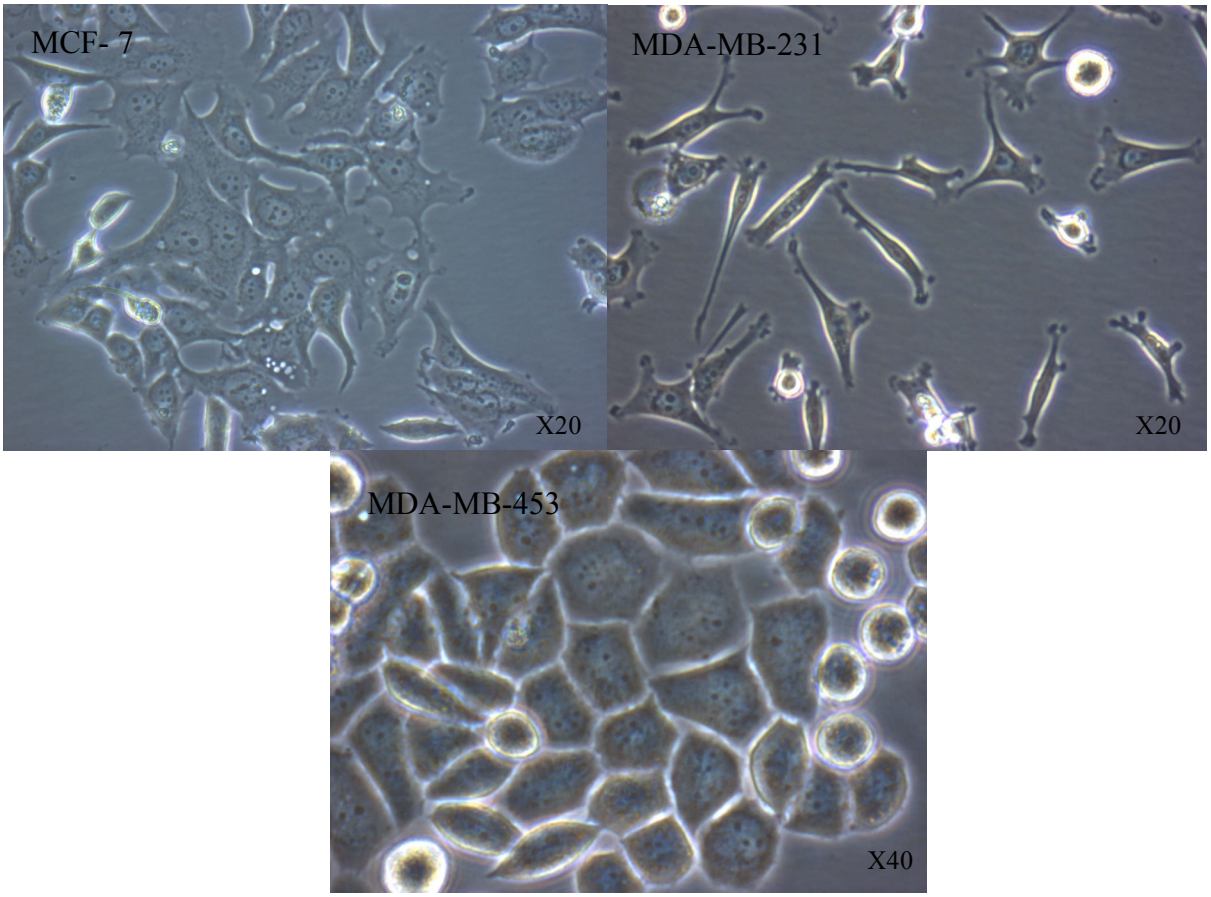

Figure 5.1: Microscopic morphology of MCF-7, MDA-MB-231, and MDA-MB-453 breast cancer cell lines in culture media.

5.1.2 ERα Immunofluorescence on MCF-7, MDA-MB-231 and MDA-MB-453.

The mouse anti-human ERα antibody was used to test for the ERα status in the MCF-7, MDA-MB-231 and MDA-MB-453 cell lines. MCF-7 cells not treated with the primary antibody were included as a negative control. The IF study confirmed that the MCF-7 cells used in this study express high levels of ERα (Figure 5.2). Both MDA-MB-231 and MDA-MB-453 showed no nuclear staining for ERα. Since there were traces of ER antibody staining outside the nuclei, which may be artifactual, Western blotting was carried out to confirm the ER status of the cell lines and to test for the specificity of the antibody used.

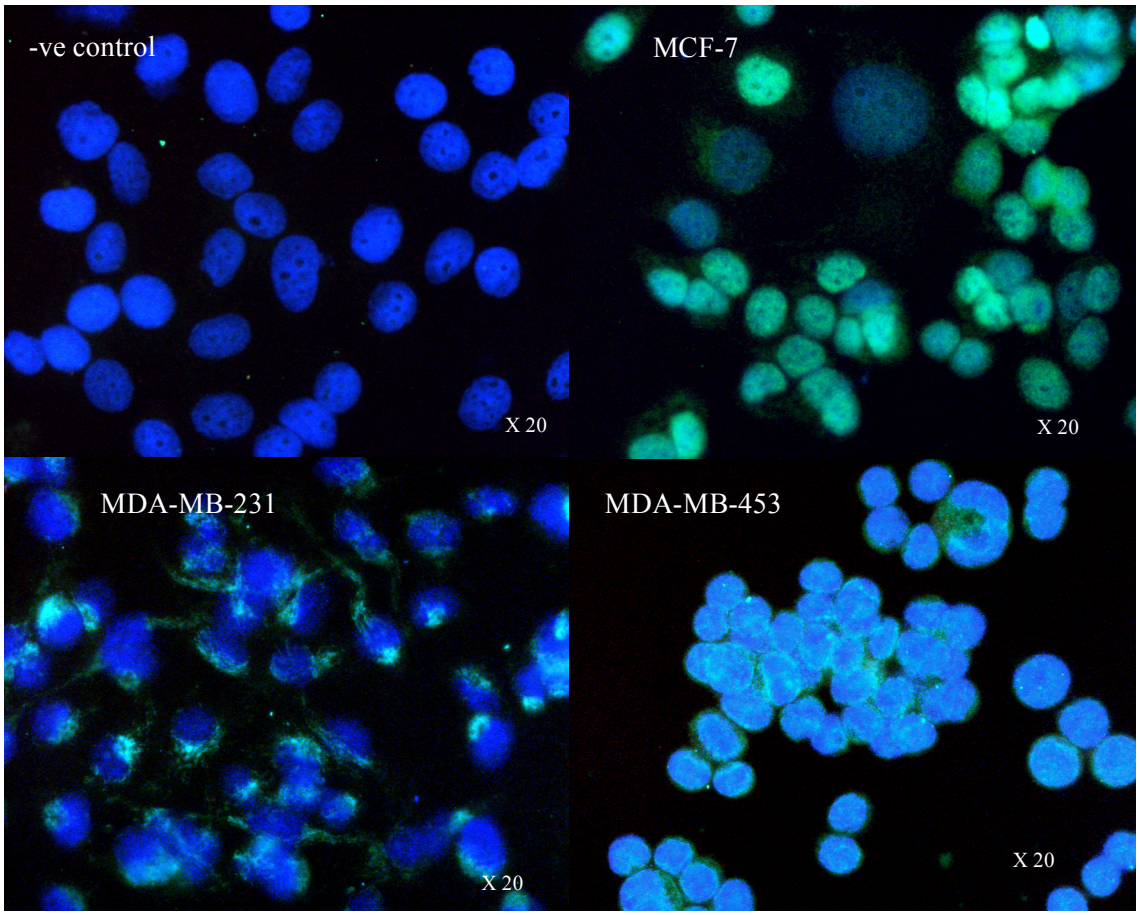

Figure 5.2. ERα: Immunofluorescence on MCF-7, MDA-MB-231 and MDA-MB-453.

Three breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) were stained with ERα antibody (1:250). A negative control (MCF-7) cells not treated with the primary antibody) was included in this study. MCF-7 showed a positive staining for ERα. Both MDA-MB-231 and MDA-MB-453 did not stain for ERαin the nuclei.

5.1.3 Western blotting for ERα in MCF-7, MDA-MB-231 and MDA-MB-453 cell lines.

Protein extracted from three different breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) was probed for ERα (66KDa). Protein was extracted from cells at two different passages to investigate for ERα status during the period of the study. Samples from the growth medium, HBSS, and cell lysis buffer were included as negative controls. ERα was detected only in the MCF-7 cells but not in the MDA-MB-231 or the MDA-MB-453 cells. The MCF-7 cells used in this study were ER+ve between passages 12 and 46 (Figure 5. 3). The same membrane was probed for tubulin (55kDa) as a loading control, after stripping off the ERα antibody (Figure 5. 3).

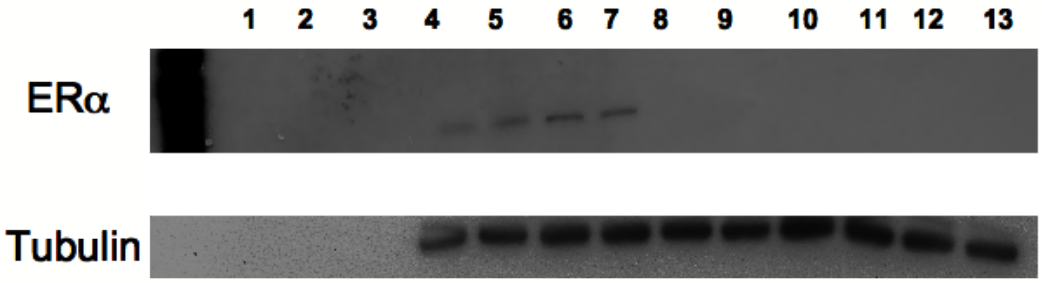

Figure 5.3: Western blotting for ERα in MCF-7, MDA-MB-231 and MDA-MB-453 cellular protein extracts.

Western blotting for protein extracted from the three studied cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-453) probed for ERα (66KDa). Protein from two different passages was probed for ERα to test for ER variation with time. ERα was detected only in the MCF-7 cells at passage 12 and passage 46. No ERα was detected in the MDA-MB-231 or the MDA-MB-453 cells. Tubulin (55kDa) was used as a loading control. Lane1: Medium, 2: HBSS, 3:cell lysis buffer, 4+ 5:MCF-7 passage 12, 6+ 7: MCF-7 Passage 46, 8+ 9: MDA-MB-453 passage 28, 10+11, MDA-MB-231 passage 49, and 11+12: MDA-MB-231passage 69

These data clearly show that the MCF-7 cell line used in this study was ERα positive and that MDA-MB-231 and MDA-MB-453 cell lines were ERα negative.

5.2 Sirtuin gene expression in response to Docetaxel treatment in an ER^{-ve} breast cancer cell line (MDA-MB-231).

5.2.1 Introduction:

Docetaxel and paclitaxel belong to Taxane anti-cancer agent, a relatively new anticancer drugs group, are classified as mitotic poisons that are commonly used as cytotoxic agents in treating metastatic and locally advanced breast cancers especially ER $^{-ve}$ tumours. Docetaxel binds to and stabilizes the β−tubulin subunit of microtubules, preventing depolymerization of the mitotic spindle thus leading to cell cycle arrest and apoptosis. (77, 78).

Although, the effect of Taxane (paclitaxel) is mediated by its interaction with microtubules causing mitotic arrest, apoptosis and different modes of cellular death have also been implicated in the Taxanes treatment. Consequently, this may lead to the variation in cellular sensitivity to Taxanes. Moreover, the mechanism by which microtubule-interfering agents induce cell death is complicated and not fully understood.

Docetaxel, a semi-synthetic analogue of paclitaxel, is a microtubule-stabilizing taxane which has recently been approved for use in the clinic for the treatment of breast and prostate cancers and small cell carcinoma of the lung (353). Since the mechanism by which microtubule-interfering agents induce cell death is poorly understood, factors involved in ageing might also be involved in mediating response to such anticancer treatments. As previously discussed the sirtuins are implicated in both the ageing process and cancer development and progression, possibly through their involvements in the MTR trinity. Therefore this study aimed to investigate sirtuin expression in an ER negative breast cancer cell line (MDA-MB-231) in response to Docetaxel treatment.

5.2.2 Establishment of experimental conditions for Docetaxel treatments.

This experiment was undertaken to determine the optimal conditions for measuring sirtuin gene expression following treatment of cells with Docetaxel. An established ER^{-ve} mammary cancer cell line, MDA-MB-231, was used.

5.2.2.1 MDA-MB-231 cell number titration.

A cell titration experiment was performed first to determine the optimal number of MDA-MB-231 cells to be seeded per well in the 96-well plate for this experiment. MDA-MB-231 cells were seeded at four different numbers per well (2500, 5000, 7500, and 10000) for three different time periods of 3, 4 and 5 days. The experiment with the MDA-MB-231 cells required incubating cells with Docetaxel for 3 days. In order to prepare for a future study of the response of sirtuin genes to Tamoxifen in the MDA-MB-231 cells, the cells were also incubated for 4 and 5 days. The data showed that a cell number of between 2500 and 5000 was the best for a 3day incubation period (Figure 5.4). Therefore in the following experiments, the number of MDA-MB-231 cells to be seeded was selected depending on the incubation period in the 96-well plate.

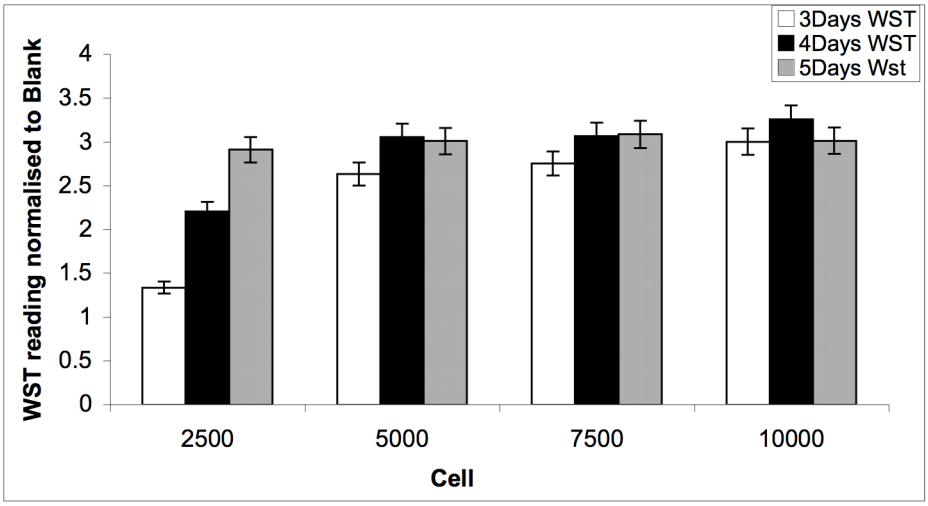

Figure 5.4: MDA-MB-231 cell titration in the 96-well plate.

5.2.2.2 Optimisation of Docetaxel concentration.

The WST proliferation assay was used to determine the conditions under which cell proliferation was strongly suppressed (50%), with minimal cytotoxic effect. Dose response experiments were performed using two different incubation times (48hrs and 72 hrs) to

determine the suitable concentration of Docetaxel. The effect of various concentrations (0.5nM, 1nM, 2nM, 5nM 10nM, 20nM, 50nM, 100nM, 1000nM) of Docetaxel on cellular proliferation were determined using the WST proliferation assay. Cell proliferation data for Docetaxel (in DMSO) treated cells are given relative to cells treated with DMSO alone (Figure 5.5a). The lower concentrations of DMSO had little effect on cell proliferation, in contrast to the higher concentrations of DMSO, which were not used in the subsequent experiments (Figure 5. 5 a). The 72 hrs incubation data are shown in (Figure 5. 5 b): 48h incubation (data not shown) was not subsequently used. The 10nM concentration of Docetaxel suppressed 50% of cellular proliferation. In order to have a range of concentrations around the 50% suppression point, concentrations of 1nM, 10nM and 100nM were selected for future experiments.

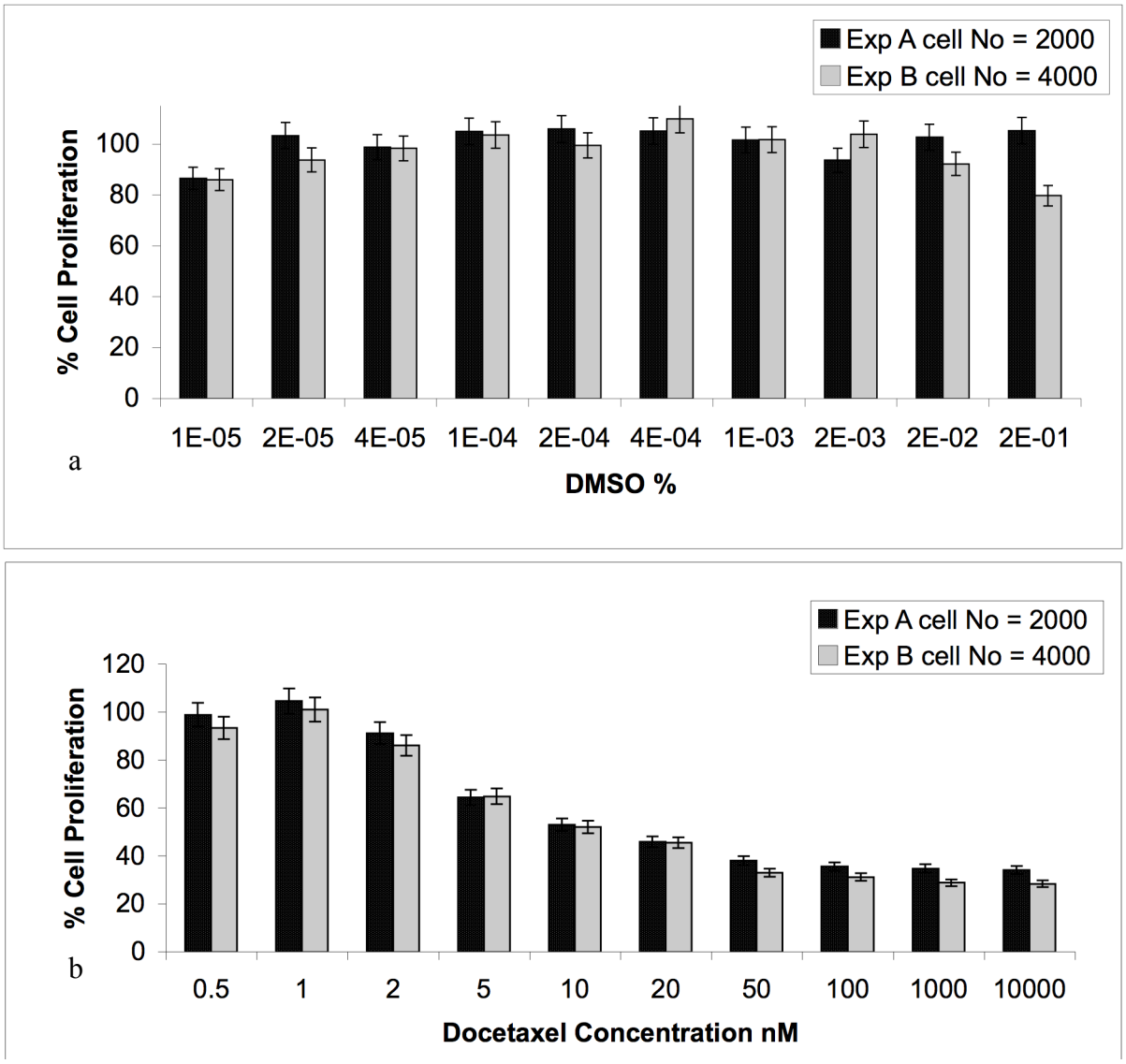

Figure 5.5: Optimisation of Docetaxel concentration for treating the MDA-MB-231 cells.

(a) DMSO effect on cell proliferation of the MDA-MB-231 cell line at 72 hrs. (b) Docetaxel dose response curve for the MDA-MB-231 breast cancer cell line.This experiment was performed in duplicate using two different cell numbers in order to investigate whether the available space for the growing cells in 96-well-plate has an effect on cellular proliferation.

5.2.2.3 Optimization of Docetaxel treatment time.

A suitable incubation time was decided upon following an experiment investigating the effects of the selected three concentrations (1nM, 10nM and 100nM) of Docetaxel at four different time points: 24hrs, 48hrs, 72hrs and 96hrs. Figure 5. 6 a shows the results of this experiment. 72hrs was chosen as a suitable incubation time because there was a significant suppression of proliferation (50%) but a sufficient number of cells remained alive to enable RNA preparation. Simultaneously, a control experiment was performed to determine the effect of the experimental concentration of DMSO on MDA-MB-231 cell proliferation. The data showed that the concentrations of DMSO used (2.E-05%, 2.E-04% and 2.E-03%) have no negative effect on MDA-MB-231 cell proliferation using the WST proliferation assay (Figure 5. 6 b).

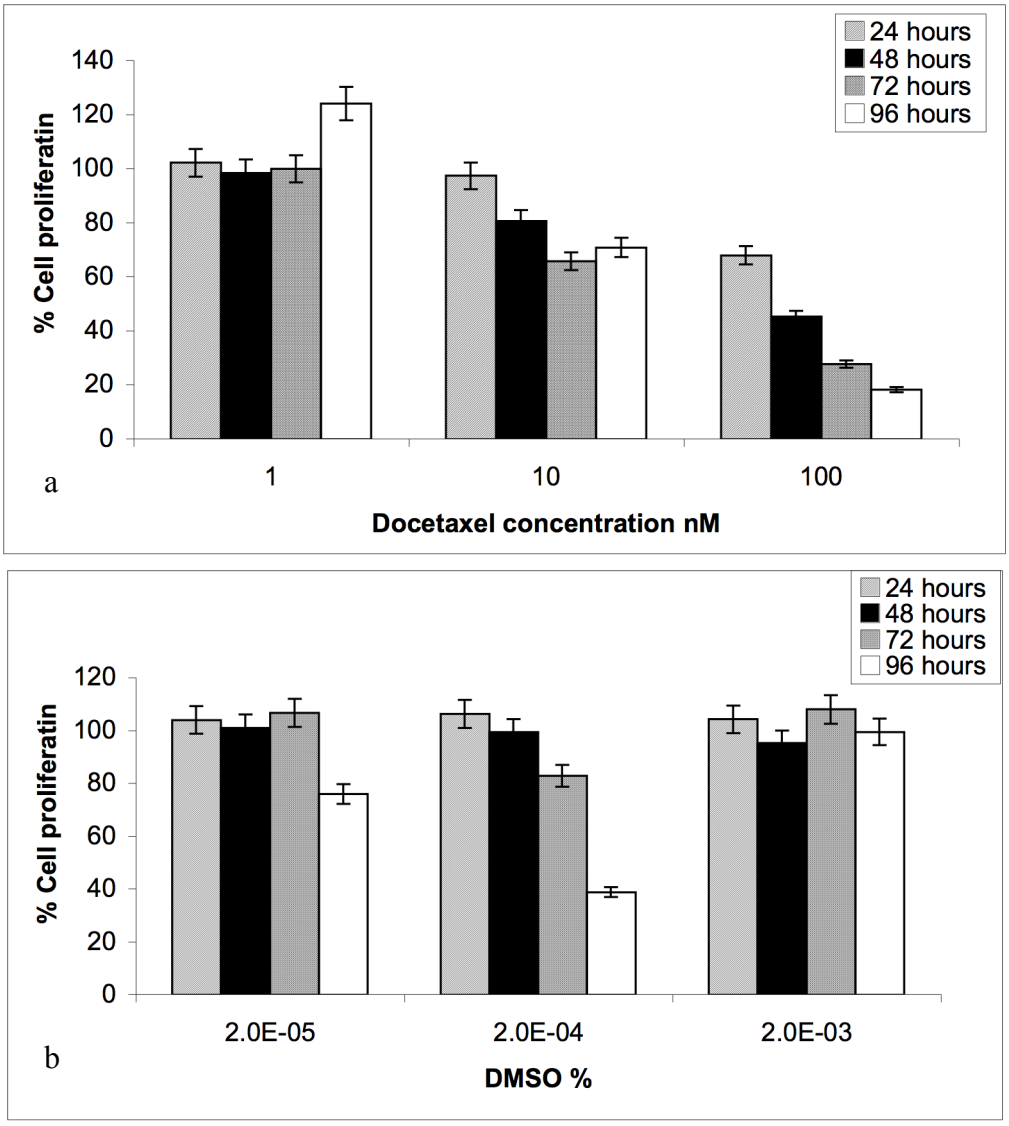

Figure 5.6: Optimization of Docetaxel treatment time in the MDA-MB-231 cell line.

(a) The MDA-MB-231 cells were incubated for various time (24hrs, 48hrs, 72hrs and 96hrs) using three different concentration of Docetaxel (1nM, 10nM and 100nM). Cell proliferation was measured using the WST assay. (b) MDA-MB-231 cellular proliferation after being treated with the experimental concentration of DMSO alone.

In all experiments described below, the MDA-MB-231 cells were therefore treated for 72 hours using the three selected concentrations of Docetaxel, at which point the RNA was prepared in order to study sirtuin gene expression. The concentration of the DMSO was adjusted to be the same in all experiment (2.E-03 (0.002%)). This concentration is the same as used in the 100 nM Docetaxel treatment described above. In each experiment a DMSO-only control was included which was used as a "Calibrator" for determining relative gene expression.

All experiments were carried out in duplicate using the same cell preparation, media and treatments. In order to allow comparison of these duplicate experiments and to identify any significant differences between them, the same calibrator was used for both experiments (control cells treated with DMSO only in experiment A). First the statistical analysis was performed for each experiment separately, then for both experiments combined together. The Analysis of variance (ANOVA) p value will be presented for each duplicate experiment separately in the graphs and in the text; whereas the detailed two-way Dunnett t-test p value will be presented for each treatment within each duplicate experiment in Table 5.1. Similarly, the Analysis of variance (ANOVA) p value will be presented for the combined experiments in the text and the detailed two-way Dunnett t-test p value for the combined data will be presented (Table 5.1).

5.2.3 Gene expression of p21, Ki67, XRCC5 and BCL2 in response to Docetaxel treatment in MDA-MB-231 cells.

Four different control genes that were expected to respond to the drug treatments were studied before analysing sirtuin gene expression, using the same RNA preparation. These genes were chosen for the following reasons; p21 is a well-known cell stress response marker; Ki67 is a marker of cellular proliferation, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) but absent in resting (G0) cells. XRCC5 is a DNA damage response marker, and BCL2 is an anti-apoptotic marker. Real time PCR was used to quantify the transcriptional expression of four control genes in relation to HPRT as a housekeeping gene as described in the Material and Methods. (Figure 5.7 a, b, c, and d) shows the relative gene expression of the four genes following Docetaxel treatment.

5.2.3.1 Separate analysis of duplicate experiments.

The control experiment showed that, as expected, 10 nM and 100 nM Docetaxel caused a significant increase in the relative gene expression of p21. This is consistent with higher levels of cellular stress being associated with Docetaxel treatment ($p < 0.001$; Table 5.1). In both experiments, there were significant changes in the relative gene expression of Ki67 following Docetaxel treatment ($p = 0.016$ for Exp. A and $p < 0.001$ for Exp. B). Interestingly, there was a significant decrease in the relative gene expression of KI67 in cells treated with 10nM ($p < 0.001$) and 100nM ($p < 0.001$) Docetaxel when compared with those treated with 1nM Docetaxel (Figure 5. 7).

Both experiments showed significant changes in the relative gene expression of XRCC5 after Docetaxel treatments ($p = 0.033$ for Exp. A and $p < 0.014$ for Exp. B). In experiment B the observed increase in gene expression was only significant with 10nM Docetaxel treatment ($p = 0.009$), whereas in experiment A the observed increase in gene expression was not significant (100nM Docetaxel treatment; $P = 0.073$; Table 5.1). However, when data from both experiments were analysed together (see below, Table 5.1), the increases in gene expression with both 10nM ($p = 0.006$) and 100nM ($p = 0.031$) Docetaxel treatment were found to be significant (Table 5.1).

There was no significant change in the level of BCL2 gene expression in response to Docetaxel treatment at any particular Docetaxel concentration (Table 5.1), however the analysis of the overall variance of the changes in BCL2 gene expression did show significance for experiment B and the combined analysis (Table 5.1).

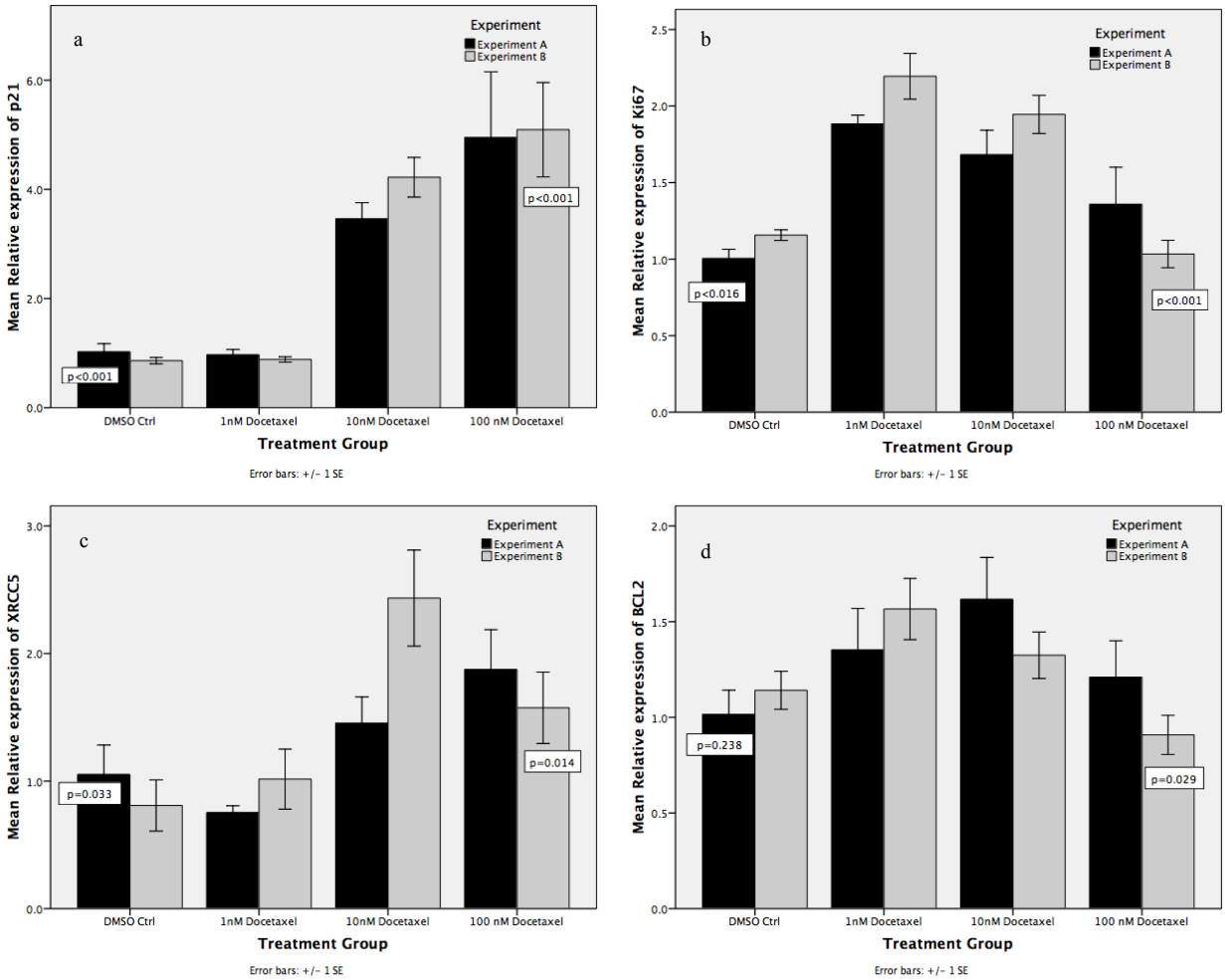

Figure 5.7: Relative transcriptional expression of p21 (a), Ki67 (b), XRCC5 (c) and BCL2 (d) genes in the MDA -MB-231 cell line in response to Docetaxel treatment.

All experiments have been done in duplicate: Exp. A dark column, and Exp. B light column.

5.2.3.2 Combined analysis of duplicate experiments.

Generalized linear model statistical analysis of both experiments together showed that there were no significant differences between the experiments for any of the control genes (p 21, p = 0.658; KI67, p = 0.340; XRCC5, p = 0.397; BCL2, p = 0.597; Table 5.1). Therefore, the combined data from both experiments can legitimately be analysed.

The combined analysis showed a significant increase in the relative transcriptional gene expression of p21 and XRCC5 after Docetaxel treatment (Figure 5. 8, $p < 0.001$ for both genes).

There was an overall significant change in the relative gene expression of KI67 ($p = 0.002$) and BCL2 ($p = 0.028$, Figure 5. 8 and Table 5.1). As can be seen from Figure 5.8 and Table 5.1, there was a trend to increased gene expression of both genes (KI67 and BCL2) at 1nM and 10nM Docetaxel concentration. However, no further increase were observed at the highest Docetaxel concentration (100nM). Instead, the relative transcriptional levels of both genes decreased at 100 nM Docetaxel treatment. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.1.

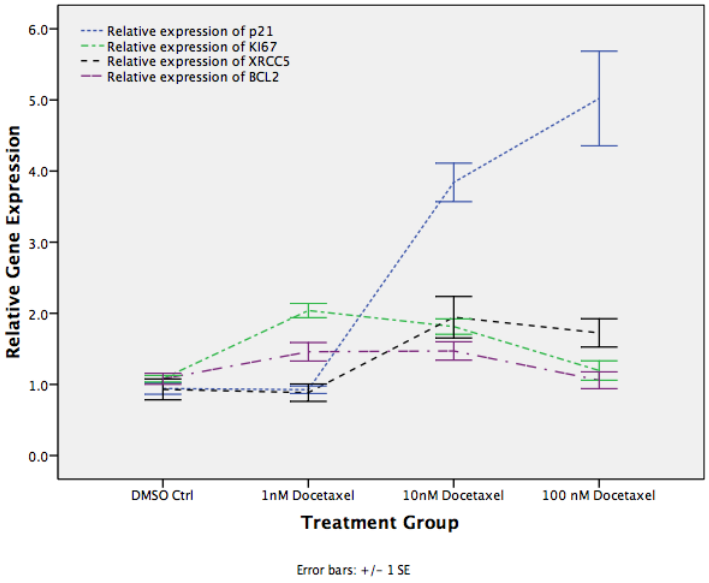

Figure 5.8: Combined data of the duplicate experiments measuring the relative transcriptional expression of p21, Ki67, XRCC5 and BCL2 genes in the MDA-MB-231 cell line in response to Docetaxel treatment.

5.2.4 SIRT1 gene expression in response to Docetaxel treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the relative transcriptional expression of SIRT1 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.9 shows the relative gene expression of SIRT1 in 1nM, 10nM and 100nM Docetaxel treated cells after 72hrs.

The data shows that there was a significant increase in relative transcriptional expression of SIRT1 gene in response to increasing concentrations of Docetaxel in MDA-MB-231 cells in both experiments ($p < 0.001$ for both Exp A and B). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.1.

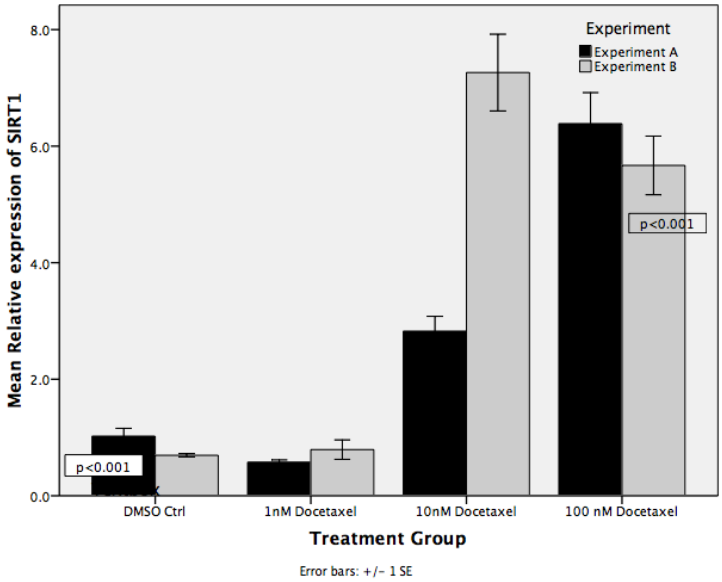

Figure 5.9: Relative transcriptional gene expression of SIRT1 in the MDA-MB-231 cell line in response to Docetaxel treatment.

A similar significant increases were observed for the relative transcriptional expression of SIRT1 gene when both experiments were combined and analysed together ($P < 0.001$, Figure 5.10). Both experiments could be combined and analysed together since the generalized linear model statistical analysis proved that there was no significant difference between the experiments ($p = 0.105$, Table 5.1).

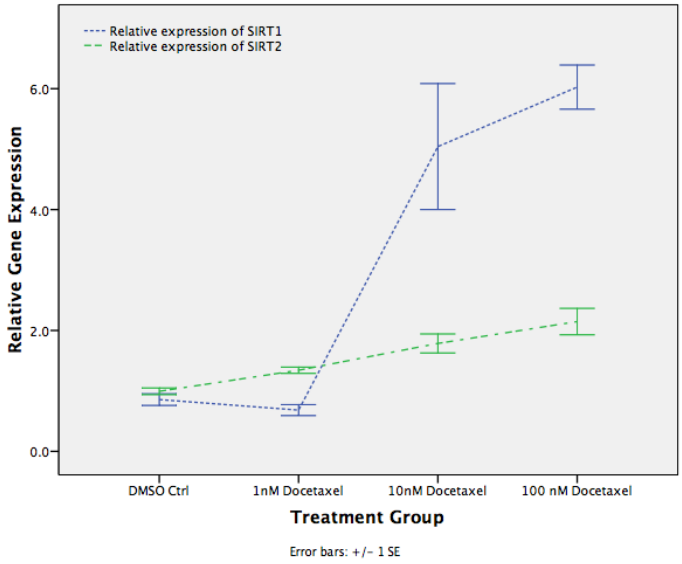

Figure 5.10: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT1 and SIRT2 genes in the MDA-MB-231 cell line in response to Docetaxel treatment.

5.2.5 SIRT2 gene expression in response to Docetaxel treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the relative transcriptional expression of SIRT2 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure

5.11 shows the relative gene expression of SIRT2 in 1nM, 10nM and 100nM cells treated with Docetaxel for 72hrs.

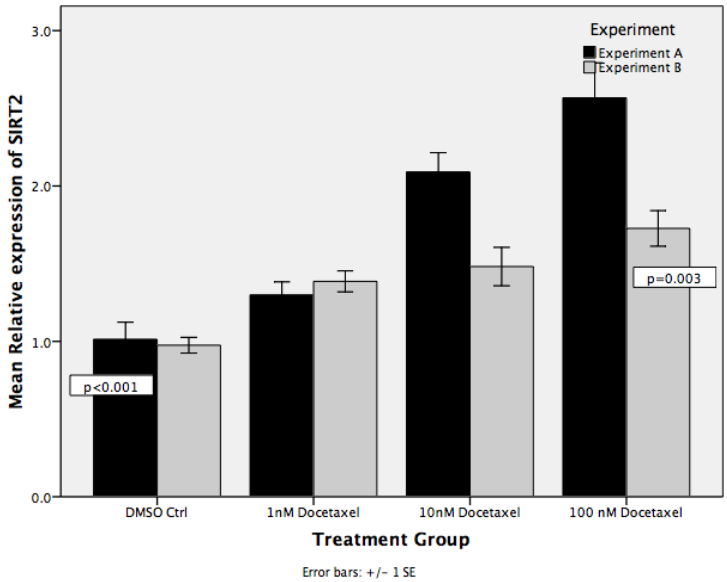

Figure 5.11: Relative transcriptional gene expression of SIRT2 in the MDA-MB-231 cell line in response to Docetaxel treatment.

Both duplicate experiments (Figure 5.11) showed that there was a significant increase in the relative transcriptional expression levels of SIRT2 gene in response to increasing concentration of Docetaxel in MDA-MB-231 cells ($p < 0.001$ for Exp. A and $p = 0.003$ for Exp. B). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.1.

Although, the generalized linear model statistical analysis proved that there was a significant difference between the duplicate experiments ($p = 0.009$, Table 5.1), a similar significant increases for relative transcriptional expression of the SIRT2 gene were observed when both experiments were combined and analysed together ($P < 0.001$, Figure 5.10).

5.2.6 SIRT3, SIRT4 & SIRT5 gene expression in response to Docetaxel treatment in MDA-MB-231 cells.

SIRT3, SIRT4 & SIRT5 are known to be mitochondrial related sirtuins and all show similar responses to Docetaxel treatment. Therefore they are presented together in this section. Real time PCR was used to quantify the transcriptional expression of SIRT3, 4 and 5 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.12 a, b, and c shows the relative gene expression of SIRT3, 4 and 5 in 1nM, 10nM and 100nM Docetaxel treated cells after 72hrs.

5.2.6.1 Separate analysis of duplicate experiments.

Our data shows that in both experiments there were significant increases in the relative transcriptional expression levels of SIRT3, SIRT4 and SIRT5 genes in response to Docetaxel treatment (in Exp. A: $p = 0.004$, $p < 0.001$ and $p = 0.005$ and in Exp. B: $p < 0.001$, $p < 0.001$ and $p = 0.001$ for SIRT3 and SIRT4 and SIRT5 respectively).

The significant increase in the relative transcriptional expression levels of SIRT3, SIRT4 and SIRT5 genes were observed mainly at the high concentrations of Docetaxel (10nM and 100nM, Figure 5.12). No reproducibly significant changes in gene expression of all three studied sirtuins were observed in response to 1 nM Docetaxel treatment. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.1.

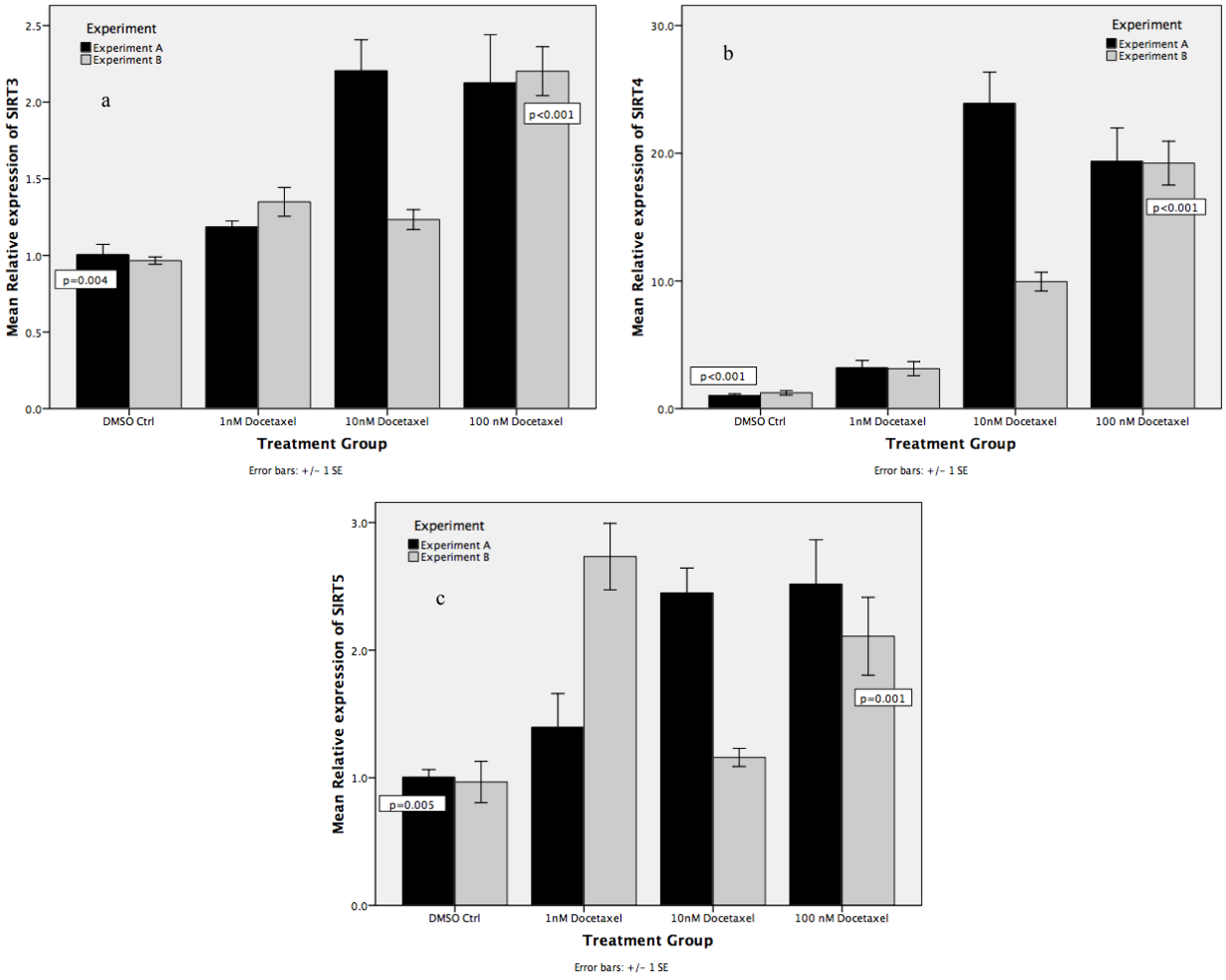

Figure 5.12: Relative transcriptional gene expression of SIRT3 (a), SIRT4 (b), and SIRT5 (c) in the MDA-MB-231 cell line in response to Docetaxel treatment.

5.2.6.2 Combined analysis of duplicate experiments.

Generalized linear model statistical analysis of both duplicate experiments combined together showed that there was no significant difference between the experiments for all three genes (SIRT3, p = 0.195; SIRT4, p = 0.052; SIRT5, p = 0.712; Table 5.1). Therefore, the combined data form both experiments can legitimately be analysed.

The combined analysis for both experiments showed significant changes in the relative transcriptional expression of SIRT3 ($p < 0.001$), SIRT4 ($p < 0.001$) and SIRT5 ($p = 0.012$) after Docetaxel treatment (Figure 5.13). There was a significant increase in the relative transcriptional expression levels of SIRT3 and SIRT4 at the high concentrations of Docetaxel (10nM and 100nM,). Whereas, the significant increase in the relative transcriptional expression levels of SIRT5 was observed only at 100 nM Docetaxel treatment. The p value from the Dunnett t-test analysis for each treatment is shown in Table 5.1.

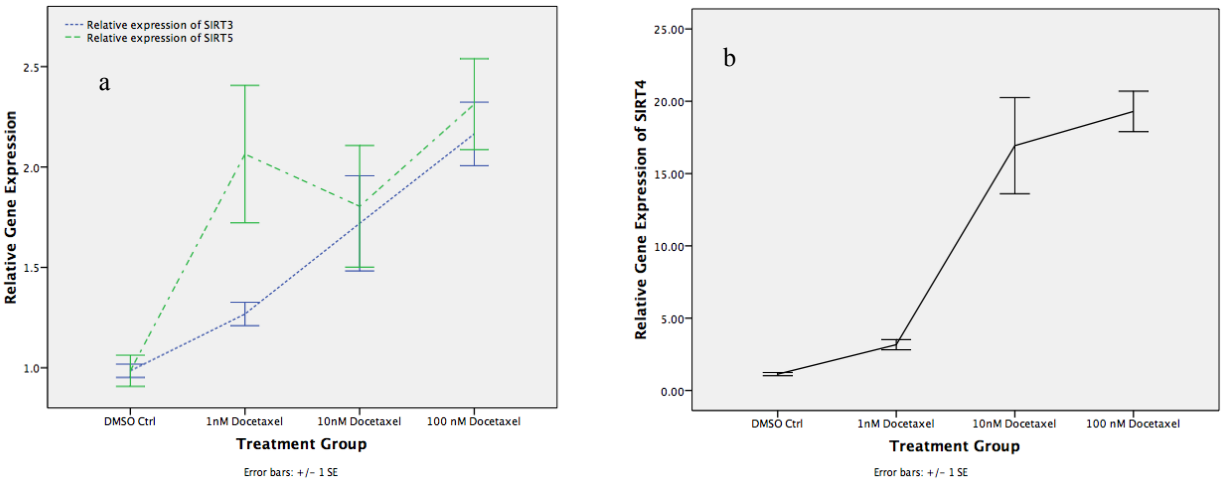

Figure 5.13: Combined data of the duplicate experiments measuring relative transcriptional gene expression of SIRT3 and SIRT5 (a) and SIRT4 (b) and in the MDA-MB-231 cell line in response to Docetaxel treatment.

5.2.7 SIRT6 gene expression in response to Docetaxel treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT6 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.14 shows the relative gene expression of SIRT6 in 1nM, 10nM and 100nM Docetaxel treated cells after 72hrs.

This experiment shows that the relative transcriptional gene expression of SIRT6 increased significantly in the MDA-MB-231 cell line when treated with 10 nM and 100 nM Docetaxel for 72 hours ($p < 0.001$ for both Exp. A and B). No significant difference in SIRT6 gene expression was observed in response to 1 nM Docetaxel (Figure 5.14). The p value for Dunnett t-test analysis for each treatment is shown in (Table 5.1).

Similar significant increases in the relative transcriptional gene expression of SIRT6 at 10nM and 100 nM Docetaxel were observed when both experiments were combined and analysed together ($P < 0.001$, Figure 5.16). Both experiments could be combined and analysed together since the generalized linear model statistical analysis proved that there was no significant difference between the experiments ($p = 0.083$, Table 5.1).

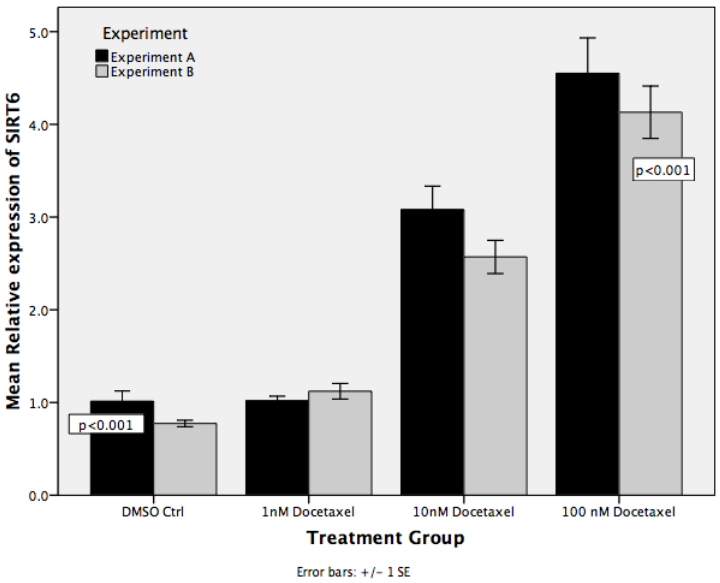

Figure 5.14: Relative transcriptional gene expression of SIRT6 in the MDA-MB-231 cell line in response to Docetaxel treatment.

5.2.8 SIRT7 gene expression in response to Docetaxel treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT7 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.15 shows the relative gene expression of SIRT7 in 1nM, 10nM and 100nM Docetaxel treated cells after 72hrs.

This experiment shows that the relative gene expression of SIRT7 increased significantly in the MDA-MB-231 cell line when treated with 10 nM and 100 nM Docetaxel for 72 hours ($p < 0.003$ for Exp. A and $p < 0.001$ for exp B). No significant difference in SIRT7

gene expression was observed in response to 1 nM Docetaxel treatment (Figure 5.15). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.1.

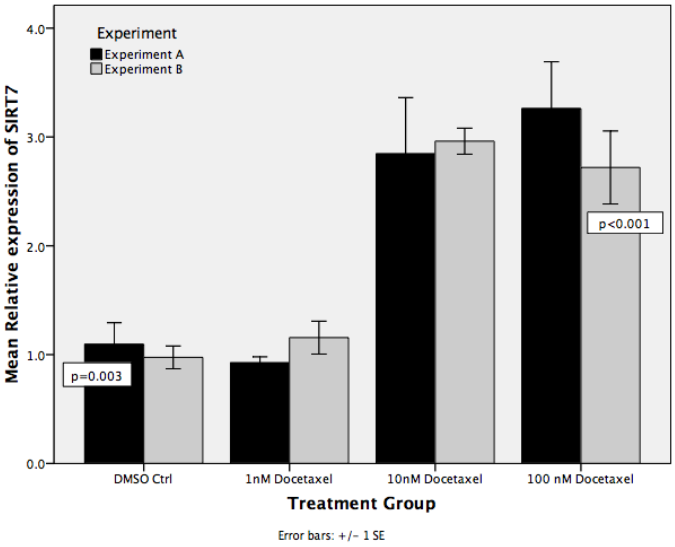

Figure 5.15: Relative transcriptional gene expression of SIRT7 in the MDA-MB-231 cell line in response to Docetaxel treatment.

Similar significant increases in the relative transcriptional gene expression of SIRT7 at 10nM and 100 nM Docetaxel were observed when both experiments were combined and analysed together ($P < 0.001$, Figure 5.16). Both experiments could be combined and analysed together since the generalized linear model statistical analysis proved that there was no significant difference between the experiments ($p = 0.697$, Table 5.1).

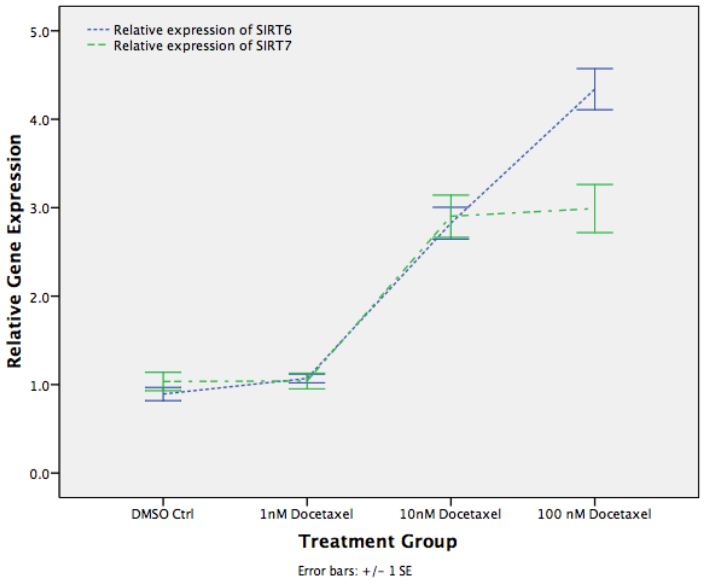

Figure 5.16: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT6 and SIRT7 genes in the MDA-MB-231 cell line in response to Docetaxel treatment.

Table 5.1: Probability levels for Docetaxel treatment experiments.

ANOVA test was used to determine the overall probability of gene expression changes as a result of Docetaxel treatment. The probability of deviation of each treatment (concentration of drug) from the control (DMSO only) was investigated using the Dunnett t-test. The statistical analysis was performed for each experiment separately and when the data form both experiments were combined together.

Experiment A			Experiment B		Combined Experiments		
P21			**P21**		**P21**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.005	p = 1.0	P < 0.001	p = 1	P < 0.001	p = 1	p = 0.658
10nM Docetaxel		p = 0.61		p = 0.003		p < 0.001	
100 nM Docetaxel		p = 0.006		p = 0.001		p = 0.001	
Ki67			**Ki67**		**Ki67**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.016	p = 0.009	P < 0.001	p < 0.001	P < 0.001	p < 0.001	p= 0.340
10nM Docetaxel		p = 0.033		p = 0.002		p = 0.002	
100 nM Docetaxel		p = 0.296		p = 0.766		p = 0.770	
XRCC5			**XRCC5**		**XRCC5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.033	p = 0.674	P = 0.014	p = 0.914	P = 0.002	p = 0.997	p = 0.397
10nM Docetaxel		p = 0.471		p = 0.009		p = 0.006	
100 nM Docetaxel		p = 0.073		p = 0.203		p = 0.031	
BCL2			**BCL2**		**BCL2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.238	p = 0.50	P = 0.029	p=0.096	P = 0.028	p = 0.085	p = 0.597
10nM Docetaxel		p = 0.135		p = 0.611		p = 0.074	
100 nM Docetaxel		p = 0.818		p = 0.444		p = 0.999	
SIRT1			**SIRT1**		**SIRT1**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P < 0.001	p = 0.625	P < 0.001	p = 0.997	p < 0.001	p = 0.997	p = 0.105
10nM Docetaxel		p = 0.008		p < 0.001		p < 0.001	
100 nM Docetaxel		p <0.001		p < 0.001		p < 0.001	
SIRT2			**SIRT2**		**SIRT2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P < 0.001	p = 0.424	P = 0.003	p = 0.037	P < 0.001	p = 0.129	p = 0.009
10nM Docetaxel		p = 0.002		p = 0.013		p < 0.001	
100 nM Docetaxel		p <0.001		p = 0.001		p < 0.001	
SIRT3			**SIRT3**		**SIRT3**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.004	p = 0.839	P < 0.001	p = 0.062	P < 0.001	p = 0.387	p = 0.195
10nM Docetaxel		p = 0.005		p = 0.207		p = 0.005	
100 nM Docetaxel		p = 0.008		p < 0.001		p < 0.001	
SIRT4			**SIRT4**		**SIRT4**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P < 0.001	p = 737	P < 0.001	p = 0.997	P < 0.001	p = 0.728	p = 0.052
10nM Docetaxel		p < 0.001		p < 0.001		p < 0.001	
100 nM Docetaxel		p< 0.001		p < 0.001		p < 0.001	
SIRT5			**SIRT5**		**SIRT5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.005	p = 0.554	P = 0.001	p = 0.001	P = 0.012	p = 0.025	p = 0.712
10nM Docetaxel		p = 0.007		p = 0.868		p = 0.104	
100 nM Docetaxel		p = 0.006		p = 0.016		p = 0.006	
SIRT6			**SIRT6**		**SIRT6**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P < 0.001	p = 1.0	P < 0.001	p = 0.404	P < 0.001	p = 0.726	p = 0.083
10nM Docetaxel		p = 0.001		p < 0.001		p < 0.001	
100 nM Docetaxel		p < 0.001		p < 0.001		p < 0.001	
SIRT7			**SIRT7**		**SIRT7**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
1nM Docetaxel	P = 0.003	p = 0.972	P < 0.001	p = 0.858	P < 0.001	p = 0.1	p = 0.697
10nM Docetaxel		p = 0.020		p < 0.001		p < 0.001	
100 nM Docetaxel		p = 0.006		p = 0.001		p = 0.001	

5.3 Sirtuin gene expression in response to Tamoxifen treatment in an ER $^{+ve}$ breast cancer cell line (MCF-7).

5.3.1 Introduction.

Endocrine treatment plays an important role in the management of hormone-dependent breast cancer. The non-steroidal antiestrogen Tamoxifen (TAM), a selective oestrogen receptor modulator (SERM) is the treatment of choice for most women with hormone receptor-positive, invasive breast carcinoma. Tamoxifen is a key treatment due to its good tolerability profile and its efficacy, both in adjuvant and first-line settings (354, 355). However, resistance to treatment is always a challenging event and it is a limiting factor in Tamoxifen treatment (356).

Tamoxifen is believed to inhibit the growth of breast cancer mainly through competing with oestrogen for oestrogen receptor binding. However, the precise mechanism by which Tamoxifen can induce apoptosis is not fully understood. Several studies have demonstrated that Tamoxifen can cause DNA damage (357), induce oxidative stress, mitochondrial dysfunction and activation of caspases (358-361). Moreover, Tamoxifen can induce apoptosis by ER-dependent and ER-independent mechanisms (360, 361).

Given the well-known involvement of sirtuins in DNA damage repair and the link between sirtuins and the mitochondrion (2), it is reasonable to propose a role for sirtuins in the mechanism of action of Tamoxifen. To address this question, we decided to investigate whether the relative transcriptional levels of sirtuin genes are altered in response to Tamoxifen treatment in both ER^{+ve} and ER^{-ve} cell lines.

5.3.2 Establishment of experimental conditions for Tamoxifen treatments

This experiment was undertaken to determine the optimal conditions for measuring sirtuin gene expression following treatment of cell lines with Tamoxifen. An established ER positive mammary cancer cell line, MCF 7 was used.

5.3.2.1 MCF-7 cell number titration.

A cell titration experiment was performed first to determine the optimal number of MCF-7 cells to be seeded per well in the 96-well plate for this experiment. MCF-7 cells were seeded at three different numbers per well (5000, 10000, and 15000) for three different time periods of 3, 4, and 5 days. The experiment with the MCF-7 cells involve a maximum of 5 days incubation in the 96-well plate; 2 days in Oestrogen free media and then a maximum of three days with the drug. The data showed that cell number between 5000 and 10000 were the best for a 5 day incubation period in the 96-well plate (Figure 5.17). Cell proliferation was limited by the well size when large cell numbers were seeded, consequently when 20000 cells were seeded it was deemed inappropriate to measure proliferation on day 5. Therefore in the following experiments, the number of MCF-7 cells to be seeded was decided depending on the incubation period in the 96-well plate.

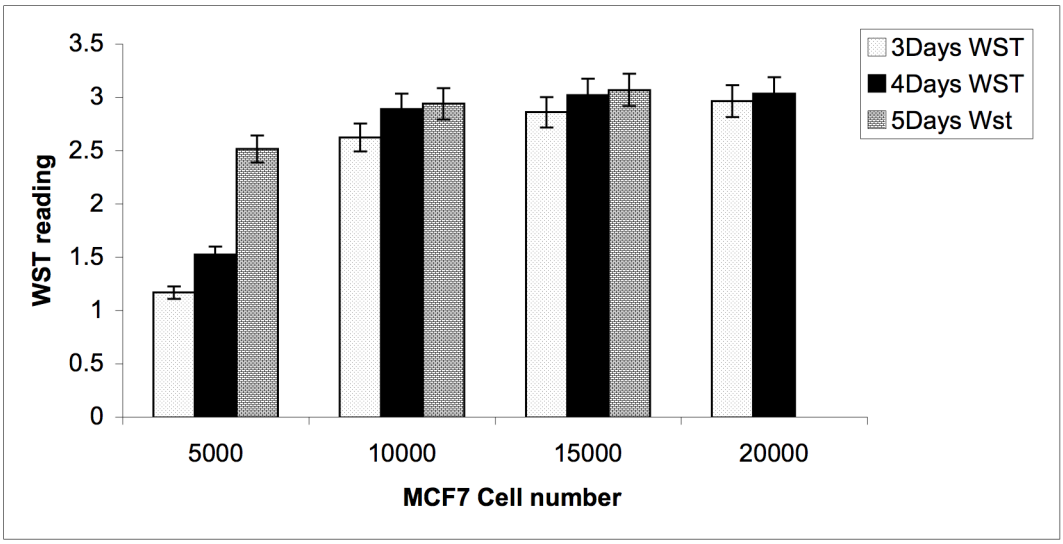

Figure 5.17: MCF-7 cell titration in the 96-well plate.

5.3.2.2 Optimisation of Tamoxifen concentration in the MCF-7 cell line.

The WST proliferation assay was used to determine the conditions under which cell proliferation was strongly suppressed (50%), with minimal cytotoxic effect. MCF-7 cells were incubated in phenol-free media supplemented with 5% charcoal stripped foetal calf serum for 48h before being treated with Tamoxifen for the tested period, to eliminate the effect of oestrogen on cellular growth (Refer to Materials and Methods). However, growth media was supplemented with oestrogen in the presence of tamoxifen, to a final concentration of 1nM.

The effects of various concentrations (2 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM and 12 μM) of Tamoxifen on cellular proliferation were determined using the WST proliferation assay. Cell proliferation data for cells treated with Tamoxifen (in DMSO) are given relative to those cells treated only with DMSO. The concentration of DMSO used had in little effect on cell proliferation (Figure 5.18a). The dose-response data for cells treated with Tamoxifen for 72 hours are shown in Figure 5.18b.

The 8 μM concentration of Tamoxifen suppressed 50% of cellular proliferation. In order to have a range of concentrations around the 50% suppression point, concentrations of 5 μM, 8 μM and 10 μM were selected for subsequent experiments.

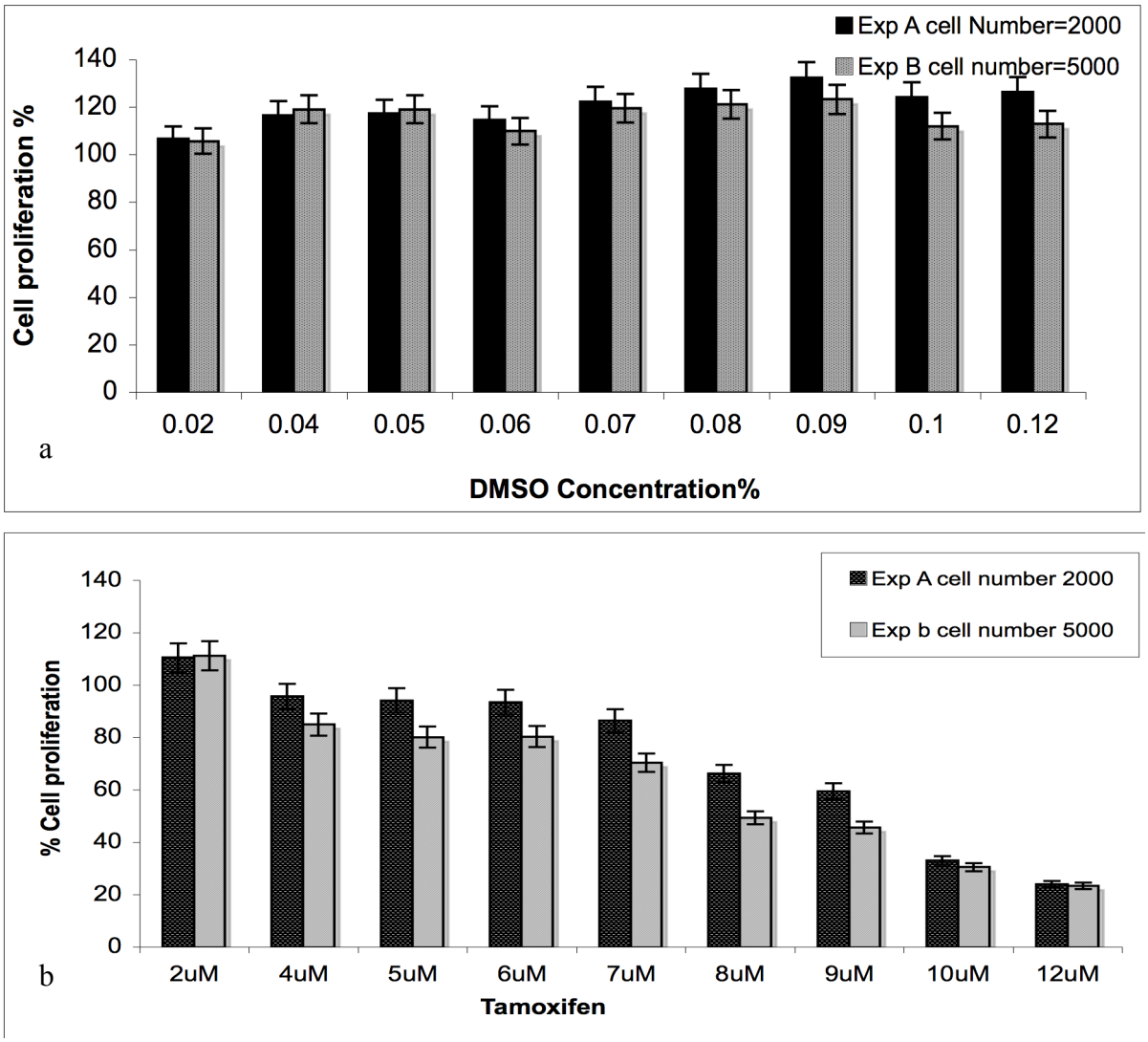

Figure 5.18: Optimisation of Tamoxifen concentration for treating MCF-7 cells.

(a) DMSO effect on cell proliferation of MCF-7 cell line at 72 hrs. (b) Tamoxifen dose response curve for the MCF-7 breast cancer cell line, This experiment was performed in duplicate using two different cell numbers (A (black column) = 2000 cells, B (grey column) = 5000 cells) in order to investigate whether the available space for the growing cells in 96-well-plate has an effect on cellular proliferation.

5.3.2.3 Optimization of Tamoxifen treatment time in the MCF-7 cell line.

A suitable incubation time was decided upon following an experiment investigating the effects of the selected three concentration (5 μM, 8 μM and 10 μM) of Tamoxifen at four different time points: 24 hrs, 48 hrs, 72 hrs and 96 hrs). Figure 5.19 a shows the results of this experiment. 72 hrs was chosen as a suitable incubation time because there was a

significant suppression of proliferation (50%) but a sufficient number of cells remained alive to enable RNA preparation at 8µM. Simultaneously, a control experiment was performed to determine the effect of the experimental concentration of DMSO on MCF-7 cell proliferation. The data showed that the concentrations of DMSO used (0.05%, 0.08% and 0.1%) have no negative effect on MCF-7 cell proliferation using the WST proliferation assay (Figure. 4.19 b).

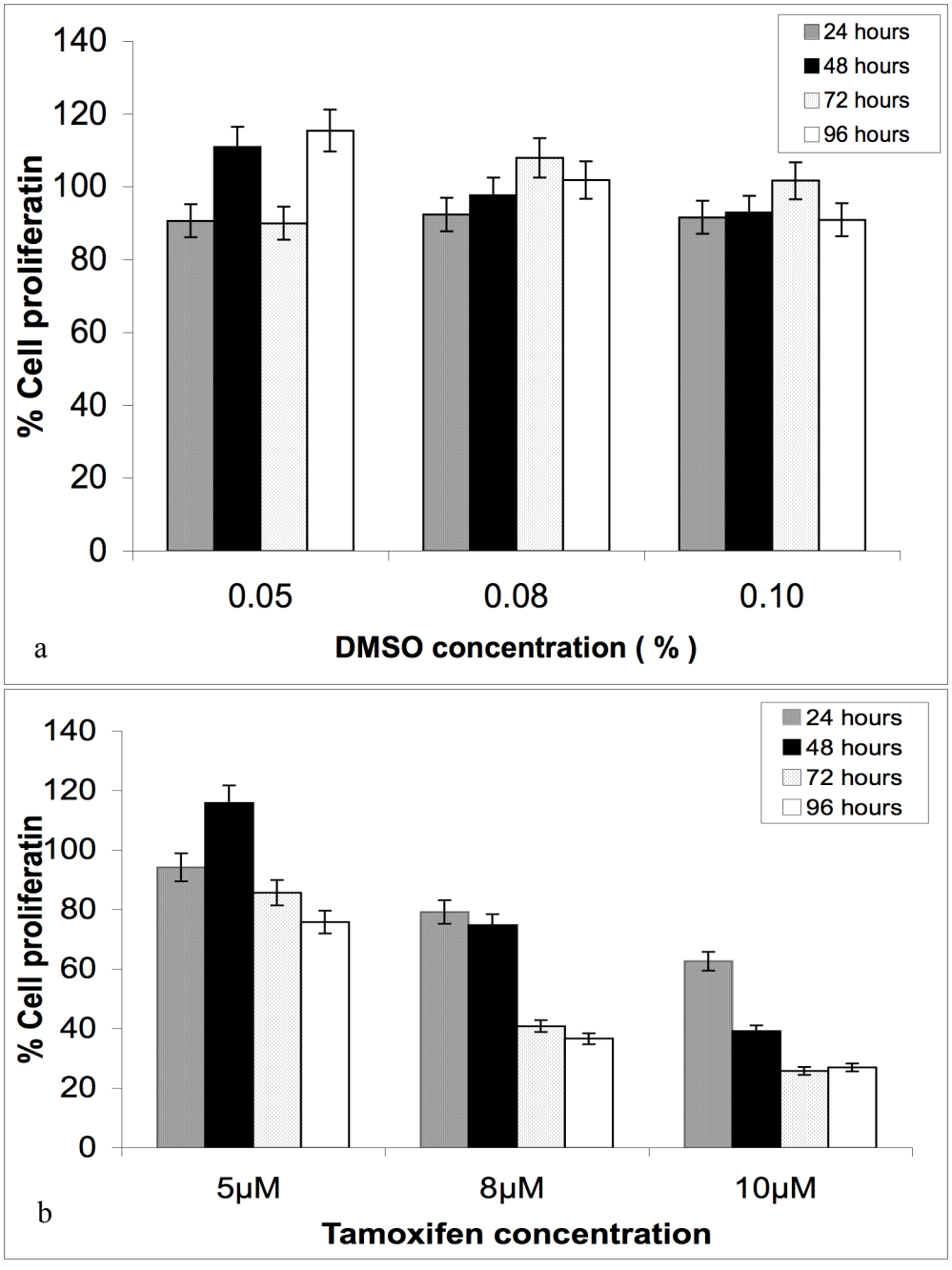

Figure 5.19. Optimization of Tamoxifen treatment time in the MCF-7 cell line.

(a) The MCF-7 cells were incubated for various time (24hrs, 48hrs, 72hrs and 96hrs) using three different concentration of Tamoxifen (5µM, 8µM and 10µM). Cell proliferation was measured using the WST assay. (b) MCF-7 cellular proliferation after being treated with the experimental concentration of DMSO alone.

In all experiments described below the MCF-7 cells were therefore treated for 72 hours using the three selected concentrations of Tamoxifen, in phenol-free media supplemented with 5% charcoal stripped fetal calf serum and 1nM β-estradiol. After 72hrs, RNA was prepared in order to study sirtuin gene expression. The concentration of the DMSO was adjusted to be the same in all experiments: 0.1%. This concentration is the same as that used in the 10 μM Tamoxifen treatment described above. In each experiment a DMSO-only control was included, which was used as a "Calibrator" for determining relative gene expression. In practice it proved that 10 μM of Tamoxifen was too cytotoxic and RNA could not be extracted successfully. Therefore in the following sections data for 5μM and 8μM only are shown.

All experiments were carried out in duplicate using the same cell preparation, media and treatments in parallel. However, the data were analysed separately for each duplicate experiment because the mathematical treatment of the RNA expression data don't allow combination of data for statistical analysis. Therefore, the p value will be presented for each duplicate experiment separately; the Analysis of variance (ANOVA) p value will be presented in the text, whereas the detailed two-way Dunnett t-test p value will be presented for each treatment within each duplicate experiment in Table 5.2.

5.3.3 Gene expression of p21, Ki67, XRCC5 and BCL2 in response to Tamoxifen treatment in MCF-7 cells.

Four different control genes that were expected to respond to Tamoxifen treatment(p21, KI67, BCL2 and XRCC5) were studied before analysing sirtuin gene expression. using the same RNA preparation. Figure 5.20 a, b, c, and d shows the relative gene expression of these genes in MCF-7 cells treated for 72 hours with 5 μM and 8 μM Tamoxifen.

5.3.3.1 Separate analysis of duplicate experiments.

This control experiment showed that, as expected, 8 μM Tamoxifen caused significant increases in the relative expression of p21 indicating higher levels of cellular stress ($p = 0.003$ and $p < 0.001$ for experiment A and B respectively, Figure 5.20 a). The relative expression of Ki67 was significantly decreased following the 8μM Tamoxifen treatment ($p < 0.001$ for experiment A and $p = 0.002$ for experiment B, Figure 5.20 b). This correlates with the anti-proliferative effect of Tamoxifen on ER^{+ve} cell lines.

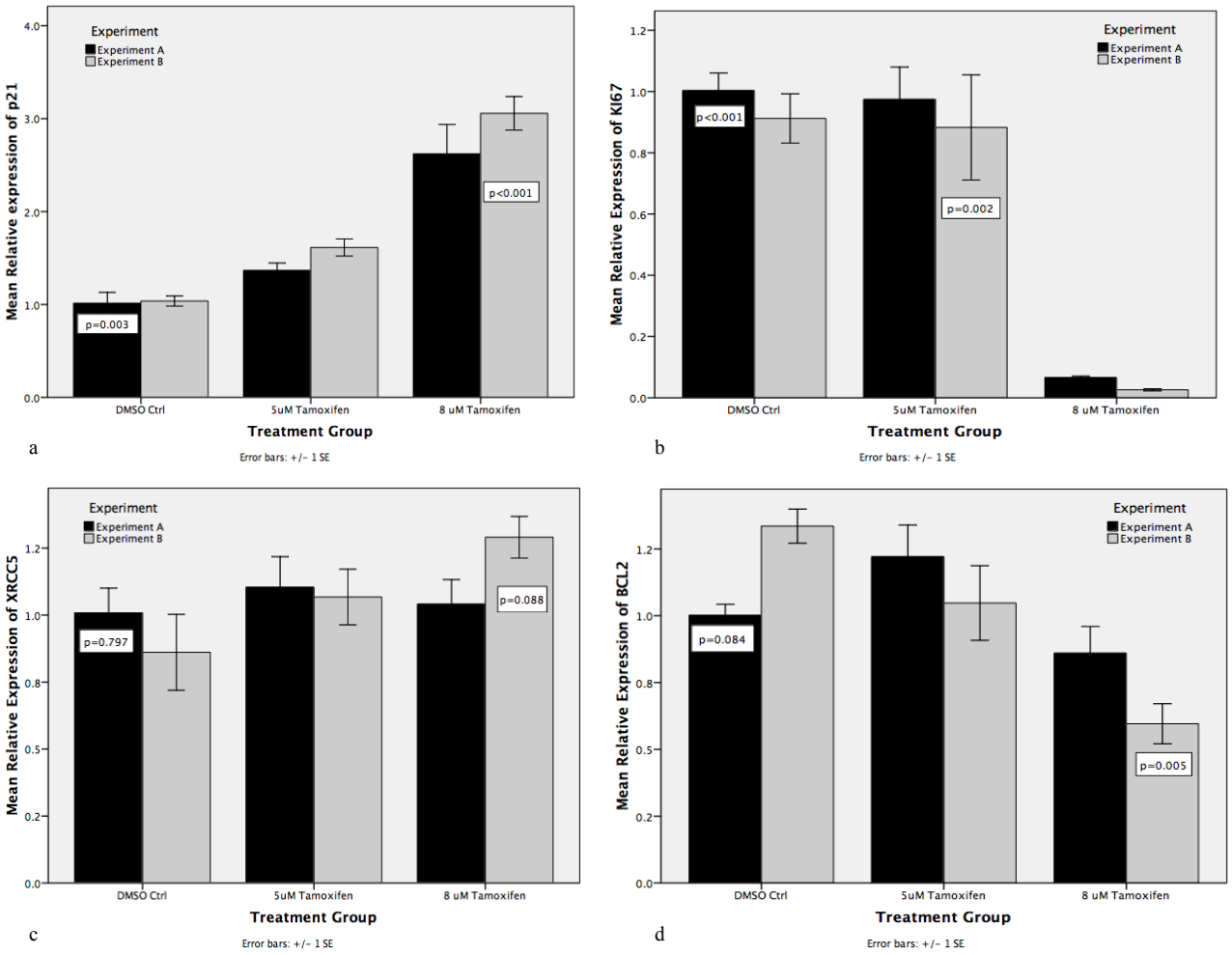

Figure 5.20: Relative transcriptional expression of p21 (a), Ki67 (b), XRCC5 (c) and BCL2 (d) genes in the MCF-7 cell line in response to Tamoxifen treatment.

All experiments have been done in duplicate: Exp. A dark column, and Exp. B light column.

Both experiments showed no significant changes in the relative transcriptional gene expression of XRCC5 after Tamoxifen treatment (P = 0.797 for Exp. A and p = 0.088 for Exp B, Figure 5.20 c).

Experiment B showed that the relative transcriptional gene expression of BCL2 decreased significantly in response to Tamoxifen treatment (p = 0.005, Figure 5.20 d). However, a non-significant decrease was observed for BCL2 gene expression following Tamoxifen treatment in experiment A (p = 0.084, Figure 5. 20 d). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

5.3.3.2 Combined analysis of duplicate experiments.

Generalized linear model statistical analysis of both duplicate experiments together showed that there was no significant difference between the experiments for all control genes (p 21, p = 0.098; KI67, p = 0.306; XRCC5, p = 0.814; BCL2, p = 0.738; Table 5.2). Therefore, the combined data from both experiments can legitimately be analysed.

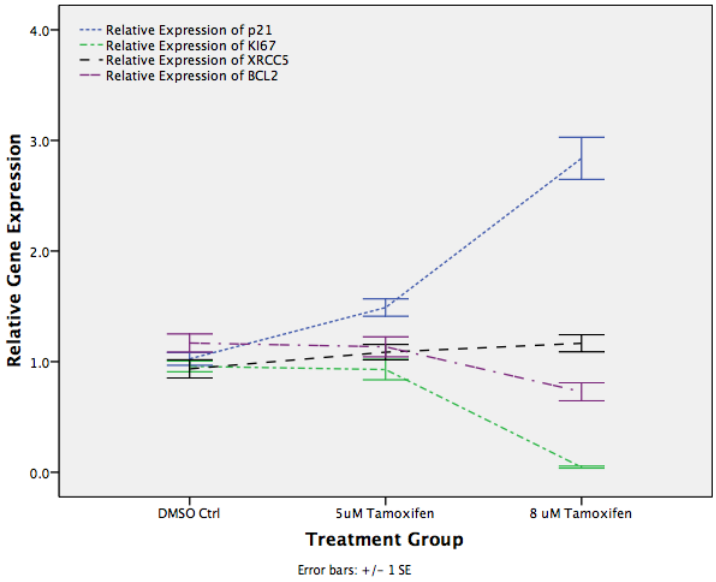

Figure 5.21: Combined data of the duplicate experiments measuring the relative transcriptional expression of p21, Ki67, XRCC5 and BCL2 genes in the MDA-MB-231 cell line in response to Tamoxifen treatment.

The combined analysis showed a significant increase in the relative transcriptional gene expression of p21 after Tamoxifen treatment (Figure 5.21, p < 0.001). However, the increase in the relative transcriptional gene expression of XRCC5 was not significant following 5μM and 8μM Tamoxifen treatment (Figure 5.21 and Table 5.2).

The combined analysis of data from both experiments showed a significant decrease in the relative transcriptional gene expression of KI67 (p < 0.001) and BCL2 (p = 0.005) after an 8μM Tamoxifen treatment (Figure 5. 21and Table 5.2).

5.3.4 SIRT1 gene expression in response to Tamoxifen treatment in MCF-7 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT1 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.22 shows the relative gene expression of SIRT1 in 5μM and 8μM Tamoxifen treated cells after 72hrs.

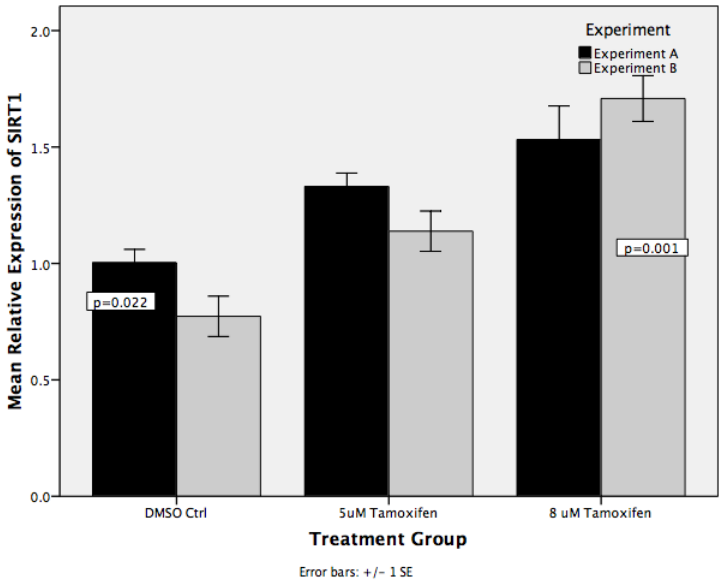

Figure 5.22: Relative gene expression of SIRT1 in the MCF-7 cell line in response to Tamoxifen treatment.

The data showed that there was a significant increase in the relative transcriptional gene expression of SIRT1 in the MCF-7 cells in response to increasing concentrations of Tamoxifen treatment ($p = 0.022$ for experiment A and $p = 0.001$ for experiment B, Figure 5. 22). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

Similar significant increases were observed when both experiments were combined and analysed together ($P < 0.001$, Figure 5. 24). Both experiments could be combined and analysed together since the generalized linear model statistical analysis proved that there was no significant difference between the experiments ($p = 0.358$, Table 5.2).

5.3.5 SIRT2 gene expression in response to Tamoxifen treatment in MCF-7 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT2 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.20 shows the relative gene expression of SIRT2 in MCF-7 cells treated with 5μM and 8μM Tamoxifen for 72 hrs.

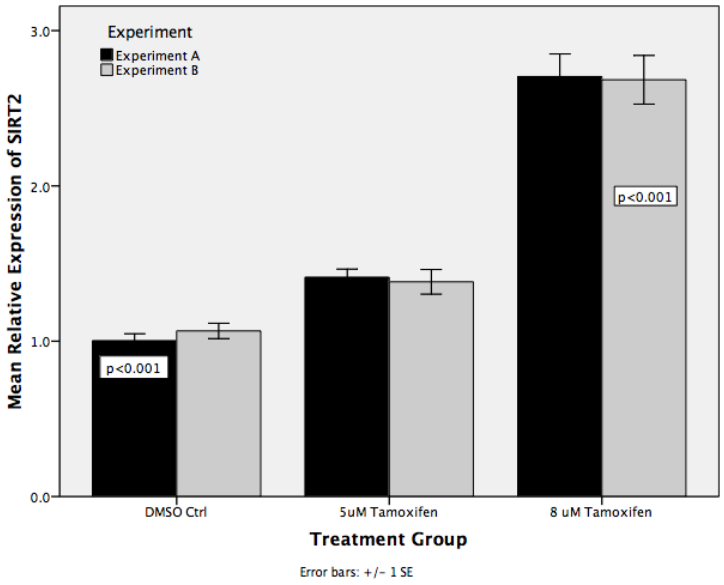

Figure 5.23: Relative transcriptional gene expression of SIRT2 in the MCF-7 cell line in response to Tamoxifen treatment.

The data shows that there was a significant increase in SIRT2 gene expression in response to increasing concentrations of Tamoxifen in the MCF-7 cells ($p < 0.001$ for both experiments; A and B, Figure 5. 23). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

The generalized linear model statistical analysis proved that there was no significant experimental difference between the duplicate experiments ($p = 0.948$, Table 5.1). The analysis of the combined data showed that there was a significant increase in the relative transcriptional gene expression of SIRT2 following Tamoxifen treatment ($P < 0.001$, Figure 5.24, Table 5.2).

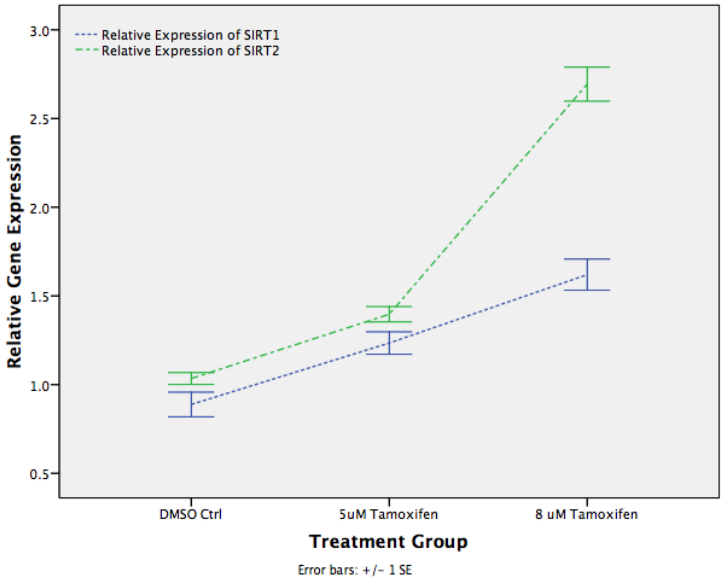

Figure 5.24: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT1 and SIRT2 genes in the MCF-7 cell line in response to Tamoxifen treatment.

5.3.6 SIRT3, SIRT4 & SIRT5 gene expression in response to Tamoxifen treatment in MCF-7 cells.

SIRT3, SIRT4 & SIRT5 are known to be mitochondrial related sirtuins and all show similar responses to Tamoxifen treatment. Therefore they are presented together in this section. Real time PCR was used to quantify the transcriptional expression of SIRT3, SIRT4 and SIRT5 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.25 a, b, and c shows the relative transcriptional expression levels of SIRT3, SIRT4 and SIRT5 genes in 5µM and 8µM Tamoxifen treated cells after 72hrs.

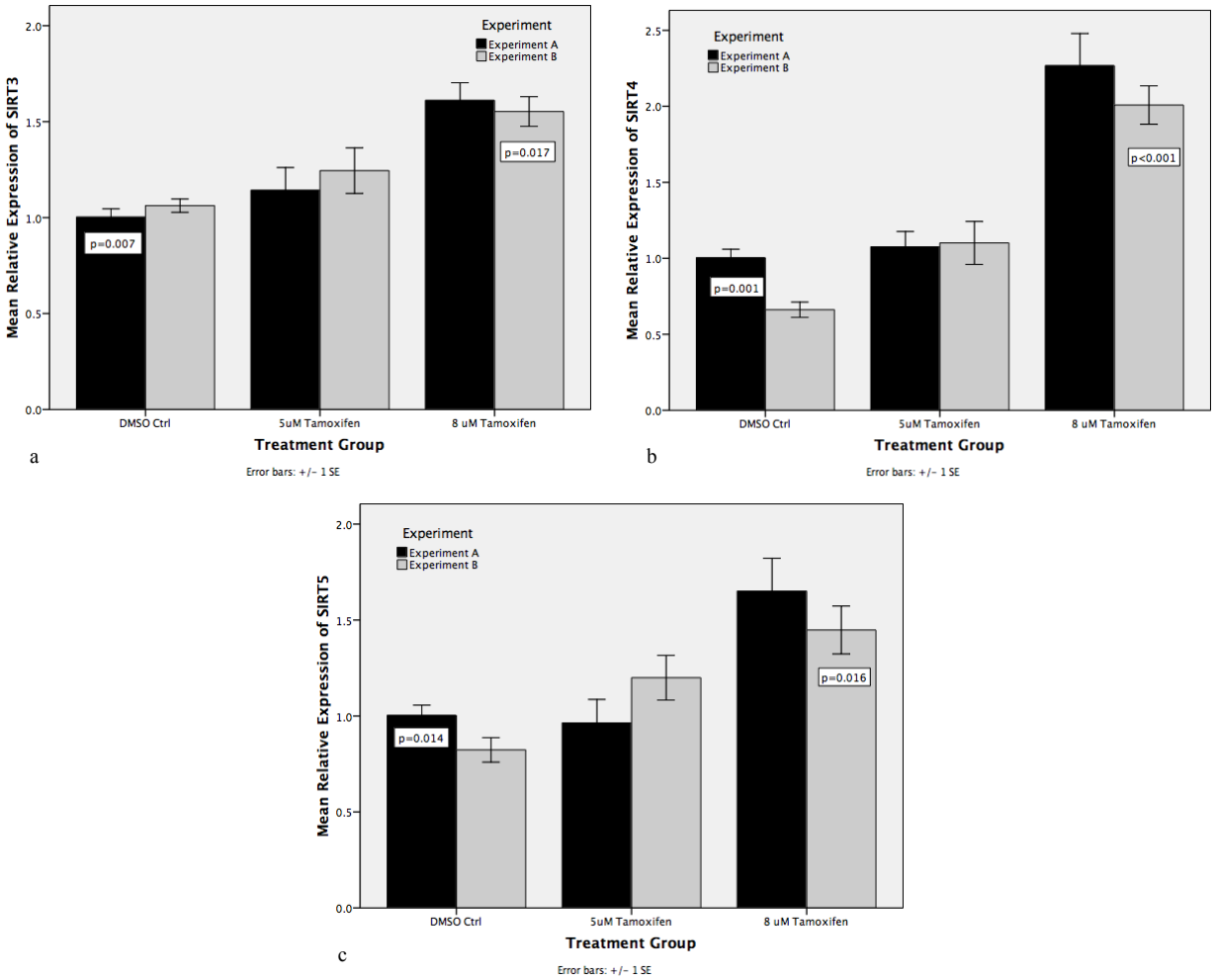

Figure 5.25: Relative transcriptional gene expression of SIRT3 (a), SIRT4 (b), and SIRT5 (c) in the MCF-7 cell line in response to Tamoxifen treatment.

Both experiment A and B showed that there was a significant increase in the relative transcriptional levels of SIRT3, SIRT4 and SIRT5 genes in response to Tamoxifen treatment ($p = 0.022$, $p = 0.007$ and $p = 0.014$ respectively for experiment A and $p = 0.017$, $p < 0.001$ and $p = 0.016$ for experiment B, Figure 5.25 a, b, and c). The significant increase

was observed following treatment with the highest concentrations of Tamoxifen treatment (8 μM). The p value for Dunnett t-test analysis is shown in Table 5.2.

Generalized linear model statistical analysis of both duplicate experiments together showed that there was no significant difference between the experiments for all three genes (SIRT3, p = 0.618; SIRT4, p = 0.09; SIRT5, p = 0.634; Table 5.2). Therefore, the combined data form both experiments can legitimately be analysed.

The combined analysis for both experiments showed significant changes in the relative transcriptional expression of SIRT3 ($p < 0.001$), SIRT4 ($p < 0.001$) and SIRT5 ($p = 0.001$) after Tamoxifen treatment (Figure 5.26). The significant increases in the relative transcriptional expression of SIRT3, SIRT4 and SIRT5 genes were only observed at the 8μM Tamoxifen concentration. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

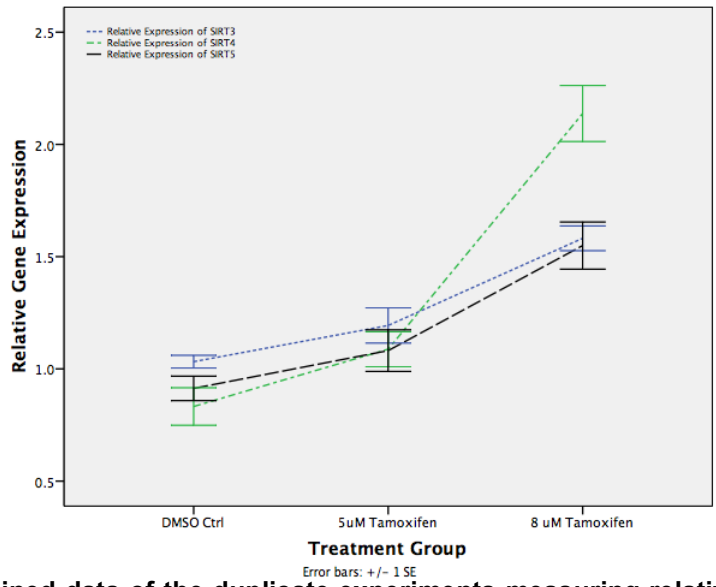

Figure 5.26: Combined data of the duplicate experiments measuring relative transcriptional gene expression of SIRT3, SIRT4 and SIRT5 in the MCF-7 cell line in response to Tamoxifen treatment.

5.3.7 SIRT6 gene expression in response to Tamoxifen treatment in MCF-7 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT6 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.27 shows the relative gene expression of SIRT6 in MCF-7 cells treated with 5μM and 8μM Tamoxifen for 72 hrs.

This experiment showed that the relative gene expression of SIRT6 increased significantly in the MCF-7 cell line when treated with 5 μM and 8 μM Tamoxifen for 72 hours ($p <$

0.001 and p = 0.002 for experiment A and B respectively, Figure 5.27). The 8μM Tamoxifen treatment showed a significant increase in both duplicate experiments. Whereas, only experiment A showed a significant increase the relative transcriptional levels of SIRT6 in the MCF-7 cells treated with 5μM Tamoxifen for 72hrs. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

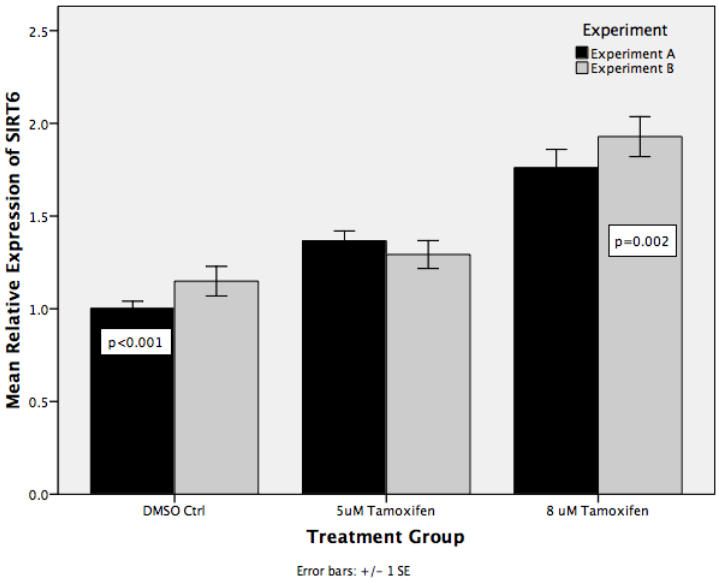

Figure 5.27: Relative gene expression of SIRT6 in the MCF-7 cell line in response to Tamoxifen treatment.

Similar significant increases in the relative transcriptional gene expression of SIRT6 at 8μM Tamoxifen were observed when both experiments were combined and analysed together (P < 0.001, Figure 5.29). Both experiments could be combined and analysed together since the generalized linear model statistical analysis proved that there was no significant difference between the experiments (p = 0.247, Table 5.2).

5.3.8 SIRT7 gene expression in response to Tamoxifen treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT7 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.28 shows the relative gene expression of SIRT7 in 5μM and 8μM Tamoxifen treated cells after 72hrs.

Both duplicate experiments showed that relative transcriptional gene expression of SIRT7 increased significantly in the MCF-7 cells when treated with Tamoxifen for 72 hrs (p = 0.004 and p = 0.016 for experiment A and B respectively, Figure 5.28). The significant increase of the relative transcriptional gene expression of SIRT7 was mainly observed

following treatment with the highest concentration of Tamoxifen (8 μM). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.2.

Although, the generalized linear model statistical analysis showed that there was significant experimental difference between the duplicate experiments ($p = 0.001$, Table 5.2), similar significant increases for relative transcriptional gene expression of SIRT7 was observed when both experiments were combined and analysed together ($P < 0.001$, Figure 5.29).

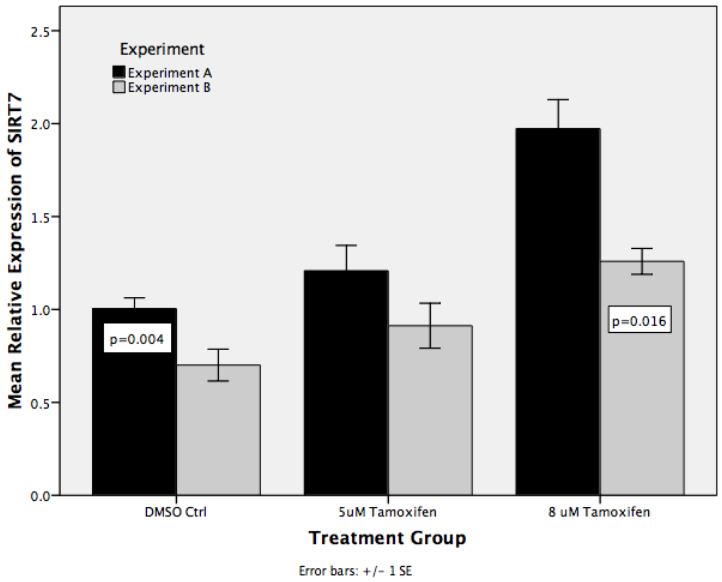

Figure 5.28: Relative gene expression of SIRT6 in the MCF-7 cell line in response to Tamoxifen treatment.

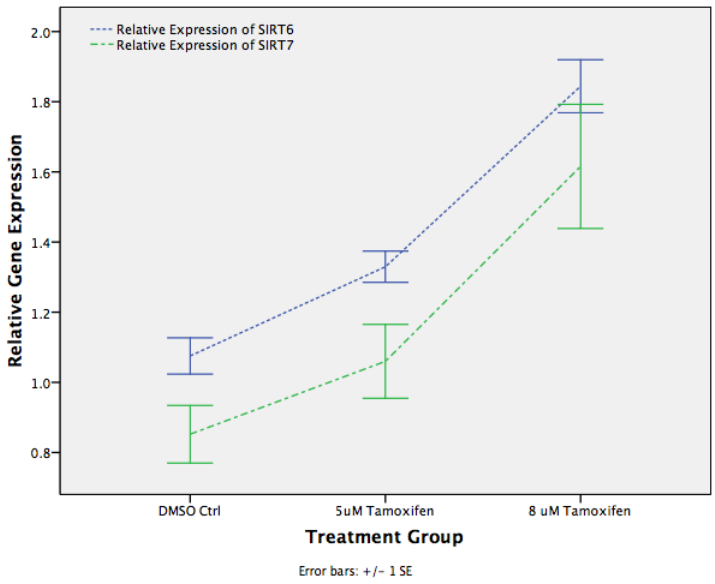

Figure 5.29: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT6 and SIRT7 genes in the MCF-7 cell line in response to Tamoxifen treatment.

Table 5.2: Probability levels for Tamoxifen treatment experiments in the MCF-7 cell line.

ANOVA test was used to determine the overall probability of gene expression changes as a result of Tamoxifen treatment. The probability of deviation of each treatment (concentration of drug) from the control (DMSO only) was investigated using the Dunnett t-test. The statistical analysis was performed for each experiment separately and when the data form both experiments were combined together.

Experiment A			Experiment B		Combined Experiments		
P21			**P21**		**P21**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	P = 0.003	p = 0.412	p < 0.001	p = 0.027	p < 0.001	p = 0.024	p = 0.098
8μM Tamoxifen		p = 0.002		p < 0.001		p < 0.001	
Ki67			**Ki67**		**Ki67**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p = 0.94	p = 0.002	p = 0.974	p < 0.001	p = 0.919	p = 0.306
8μM Tamoxifen		p < 0.001		p = 0.002		p < 0.001	
XRCC5			**XRCC5**		**XRCC5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.797	p = 0.735	p = 0.088	p = 0.375	p = 0.148	p = 0.327	p = 0.814
8μM Tamoxifen		p = 0.961		p = 0.059		p = 0.102	
BCL2			**BCL2**		**BCL2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.084	p = 0.239	p = 0.005	p = 0.143	p = 0.005	p = 0.946	p = 0.738
8μM Tamoxifen		p = 0.493		p = 0.003		p = 0.006	
SIRT1			**SIRT1**		**SIRT1**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.022	p = 0.091	p = 0.001	p = 0.051	p < 0.001	p = 0.001	p = 0.358
8μM Tamoxifen		p = 0.014		p = 0.001		p < 0.001	
SIRT2			**SIRT2**		**SIRT2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p<0.001	p = 0.038	p < 0.001	p = 0.132	p < 0.001	p = 0.003	p = 0.948
8μM Tamoxifen		p < 0.001		p < 0.001		p < 0.001	
SIRT3			**SIRT3**		**SIRT3**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.007	p = 0.485	p = 0.017	p = 0.286	p < 0.001	p = 0.131	p = 0.618
8μM Tamoxifen		p = 0.006		p = 0.011		p < 0.001	
SIRT4			**SIRT4**		**SIRT4**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.001	p = 0.911	p < 0.001	p = 0.058	p < 0.001	p = 0.118	p = 0.09
8μM Tamoxifen		p = 0.001		p < 0.001		p < 0.001	
SIRT5			**SIRT5**		**SIRT5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.014	p = 0.965	p = 0.016	p = 0.077	p = 0.001	p = 0.333	p = 0.634
8μM Tamoxifen		p = 0.019		p = 0.010		p < 0.001	
SIRT6			**SIRT6**		**SIRT6**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p = 0.017	p = 0.002	p = 0.460	p < 0.001	p = 0.186	p = 0.247
8μM Tamoxifen		p < 0.001		p = 0.001		p = 0.001	
SIRT7			**SIRT7**		**SIRT7**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.004	p = 0.456	p = 0.016	p = 0.269	p < 0.001	p = 0.186	p = 0.001
8μM Tamoxifen		p = 0.003		p = 0.010		p < 0.001	

5.4 Sirtuin gene expression in response to Tamoxifen treatment in an ER $^{-ve}$ breast cancer cell line (MDA-MB-453).

5.4.1 Introduction.

Tamoxifen (TAM) is commonly used for adjuvant therapy in breast cancer in patients with ER^{+ve} tumours and is a key drug for chemoprevention of breast cancer in high-risk women. Tamoxifen is believed to inhibit the growth of breast cancer mainly through competing with oestrogen for oestrogen receptors binding. However, we hypothesize that sirtuins might be involved in the mechanism of action of Tamoxifen treatment. In the previous experiment we proved that sirtuin gene expression was altered after treating the ER^{+ve} MCF-7 cell line with increasing concentrations of Tamoxifen. The experiment described in this section was designed to investigate whether the effect of Tamoxifen on sirtuin gene expression is mediated through the oestrogen receptors. In order to address this question, we decided to investigate whether the relative transcriptional levels of sirtuin genes are altered in response to Tamoxifen treatment in a cell line that is known to be ER^{-ve} (MDA-MB-453).

5.4.2 Establishment of experimental conditions for Tamoxifen treatments.

This experiment was undertaken to determine the optimal conditions for measuring sirtuin gene expression following treatment of cell lines with Tamoxifen. An established ER negative mammary cancer cell line, MDA-MB-453 was used.

5.4.2.1 MDA-MB-453 cell number titration.

A cell titration experiment was performed first to determine the optimal number of MDA-MB-453 cells to be seeded per well in the 96-well plate for this experiment. MDA-MB-453 cells were seeded at four different numbers per well (5000, 10000, 15000 and 20000) for three different time periods of 3, 4, and 5 days. The experiment with the MDA-MB-453 cells involve a maximum of 5 days incubation in the 96-well plate; 2 days in oestrogen free media and then a maximum of three days with the drug. The data showed that cell number equal to 5000 was the best for a 5 day incubation period in the 96-well plate (Figure 5.30). Cell proliferation was affected by the well size when larger cell numbers were seeded,

consequently when 20000 cells were seeded it was not possible to measure the proliferation on day 5. Therefore in the following experiments, the number of MDA-MB-453 cells to be seeded was decided depending on the incubation period in the 96-well plate.

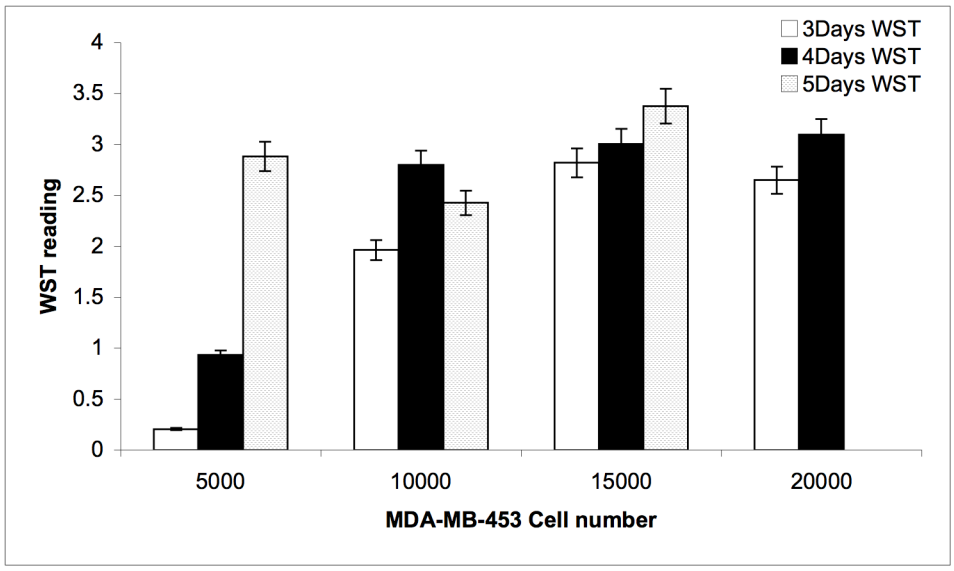

Figure 5.30: MDA-MB-453 cell titration in the 96-well plate.

5.4.2.2 Optimisation of Tamoxifen concentration.

The WST proliferation assay was used to determine the conditions under which cell proliferation was strongly suppressed (50%), with minimal cytotoxic effect. MDA-MB-453 cells was incubated in phenol-free media supplemented with 5% charcoal stripped foetal calf serum for 48h before being treated with Tamoxifen for the tested period to eliminate the effect of oestrogen on cellular growth. However, growth media was supplemented with oestrogen in the presence of Tamoxifen, to a final concentration of 1nM.

The effects of various concentrations (2 μM, 4 μM, 5 μM, 6 μM, 7 μM, 8 μM, 9 μM, 10 μM and 12 μM) of Tamoxifen on cellular proliferation were determined using the WST proliferation assay. Cell proliferation data for cells treated with Tamoxifen (in DMSO) are given relative to cells treated with DMSO alone. The concentration of DMSO used had little effect on cell proliferation (Figure. 4. 31b). The dose-response data for 72 h incubation with Tamoxifen are shown in Figure. 4. 31a.

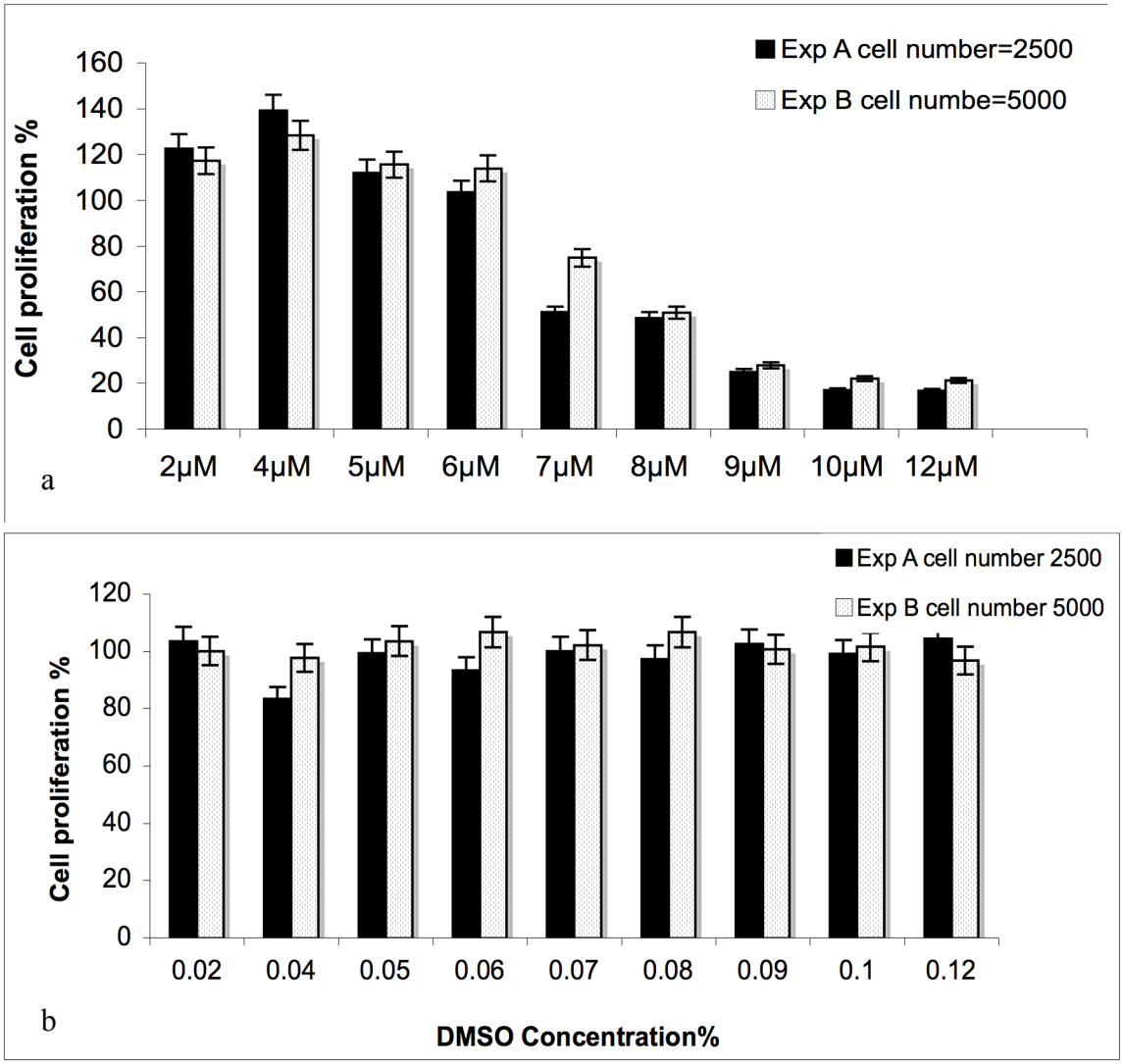

Figure 5.31. Optimisation of Tamoxifen concentration for treating MDA-MB-453 cells

The MDA-MB-453 cells were incubated for various time (24hrs, 48hrs, 72hrs and 96hrs) using three different concentration of Tamoxifen (5μM, 8μM and 10μM). Cell proliferation was measured using the WST assay. b) MDA-MB-453 cellular proliferation after being treated with the experimental concentration of DMSO alone.

The 8μM concentration of Tamoxifen suppressed 50% of cellular proliferation. In order to have a range of concentrations around the 50% suppression point, concentrations of 5μM, 8μM and 10μM were selected for subsequent experiments.

5.4.2.3 Optimization of Tamoxifen treatment time in the MDA-MB-453 cell line.

The same incubation period of 72 hrs with Tamoxifen was chosen for MDA-MB-453 cells as for the MCF-7 cells, since the response to 72hrs incubation with increasing dose of the Tamoxifen was similar for both MDA-MB-453 and MCF-7 cells (Figure 5.32). Figure 5.32a shows the effect of 72hrs incubation with three different concentrations of Tamoxifen (5 μM, 8 μM and 10 μM), and demonstrates that treating the MDA-MB-453 cells with 8 μM of Tamoxifen for 72hrs resulted in a significant suppression of proliferation (50%), and that a sufficient number of cells remain alive to enable RNA preparation. Simultaneously, a control experiment was performed to determine the effect of the experimental concentration of DMSO on MDA-MB-453 cell proliferation. The data showed that the concentrations of DMSO used (0.05%, 0.08% and 0.1%) had no negative effect on MDA-MB-453 cell proliferation using the WST proliferation assay (Figure. 4.32 b).

In all experiments described below the MDA-MB-453 cells were therefore treated for 72 hours using the three selected concentrations of Tamoxifen, in phenol-free media supplemented with 5% charcoal stripped fetal calf serum and 1nM and 1nM β-estradiol. After 72hrs, RNA was prepared in order to study sirtuin gene expression. The concentration of the DMSO was adjusted to be the same in all experiments: 0.1%. This concentration is the same as that used in the 10μM Tamoxifen treatment described above. In each experiment a DMSO-only control was included which was used as a "Calibrator" for determining relative gene expression. In contrast to the MCF-7 cells the highest concentration of Tamoxifen was not too cytotoxic for the MDA-MB-453 and RNA was extracted successfully from cells treated with 10μM Tamoxifen.

All experiments were carried out in duplicate using the same cell preparation, media and treatments in parallel. However, the data were analysed separately for each duplicate experiment because the mathematical treatment of the RNA expression data don't allow combination of data for statistical analysis. Therefore, the p value will be presented for each duplicate experiment separately; the Analysis of variance (ANOVA) p value will be presented in the text, whereas the detailed two-way Dunnett t-test p value will be presented for each treatment within each duplicate experiment in Table 5.2.

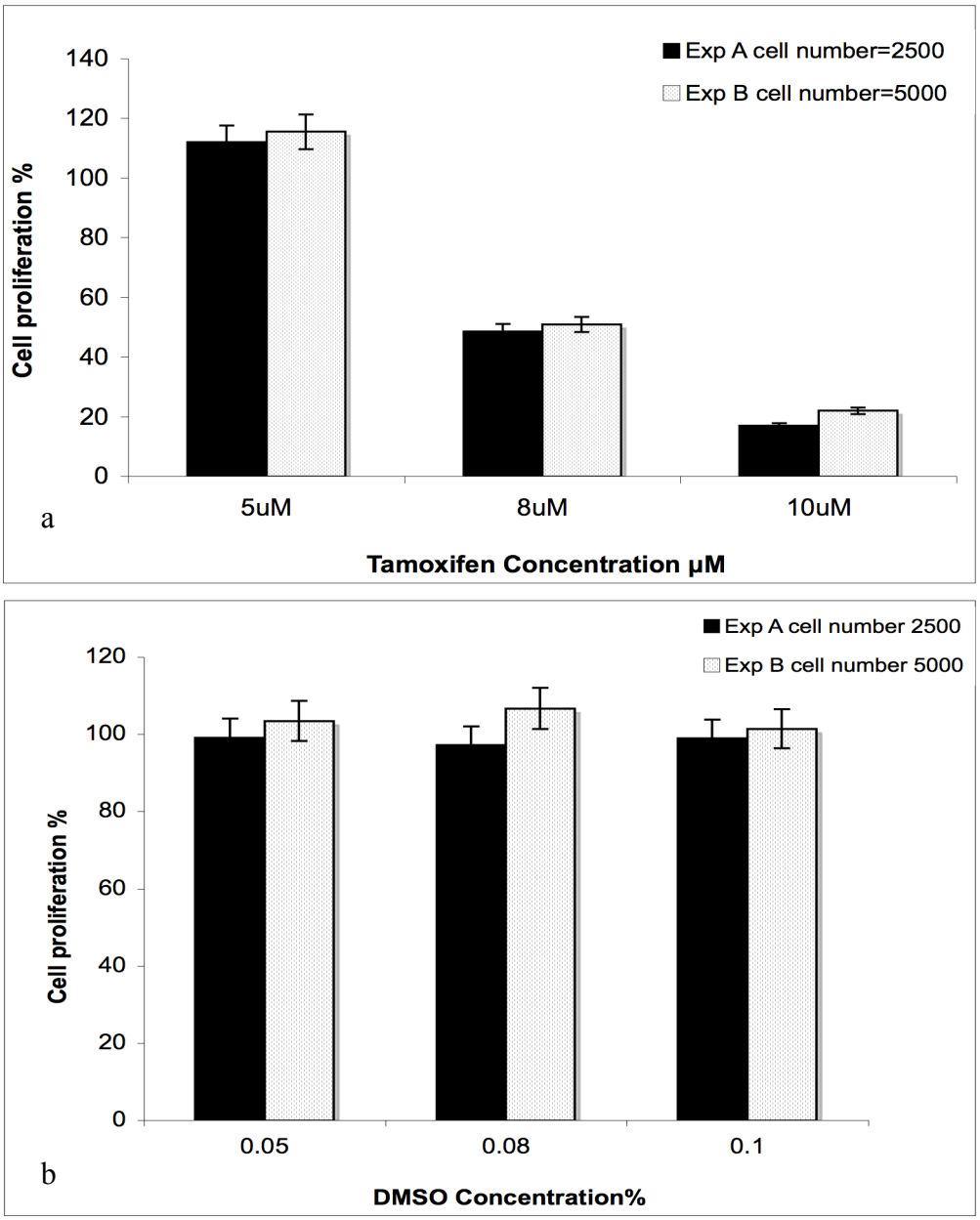

Figure 5.32: Optimization of Tamoxifen treatment time in the MDA-MB-453 cell line.

(a) The MDA-MB-453 cells were incubated for 72hrs using three different concentration of Tamoxifen (5µM, 8µM and 10µM). Cell proliferation was measured using the WST assay. (b) MDA-MB-453 cellular proliferation after being treated with the experimental concentration of DMSO alone for 72hrs.

5.4.3 Gene expression of p21, Ki67, XRCC5 and BCL2 in response to Tamoxifen treatment in MDA-MB-453 cells.

Four different control genes that were expected to respond to Tamoxifen treatments (p21, KI67, BCL2 and XRCC5) were studied before analysisng the sirtuin gene expression using the same RNA preparation. Figure 5.33 a, b, c, and d shows the relative transcriptional

expression of these genes in MDA-MB-453 cells treated for 72 hours with 5μM and 8μM Tamoxifen.

5.4.3.1 Separate analysis of duplicate experiments.

This control experiment showed that, as expected, 8μM and 10 μM Tamoxifen caused a significant increase in the relative transcriptional gene expression of p21 indicating higher levels of cellular stress ($p < 0.001$ for both experiment A and B, Figure 5.33 a). In both experiments the relative expression of Ki67 was significantly decreased following Tamoxifen treatment at all concentrations used ($p < 0.001$ for both experiment A and B; Figure 5.33 b). This correlates with its known anti-proliferative effect. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

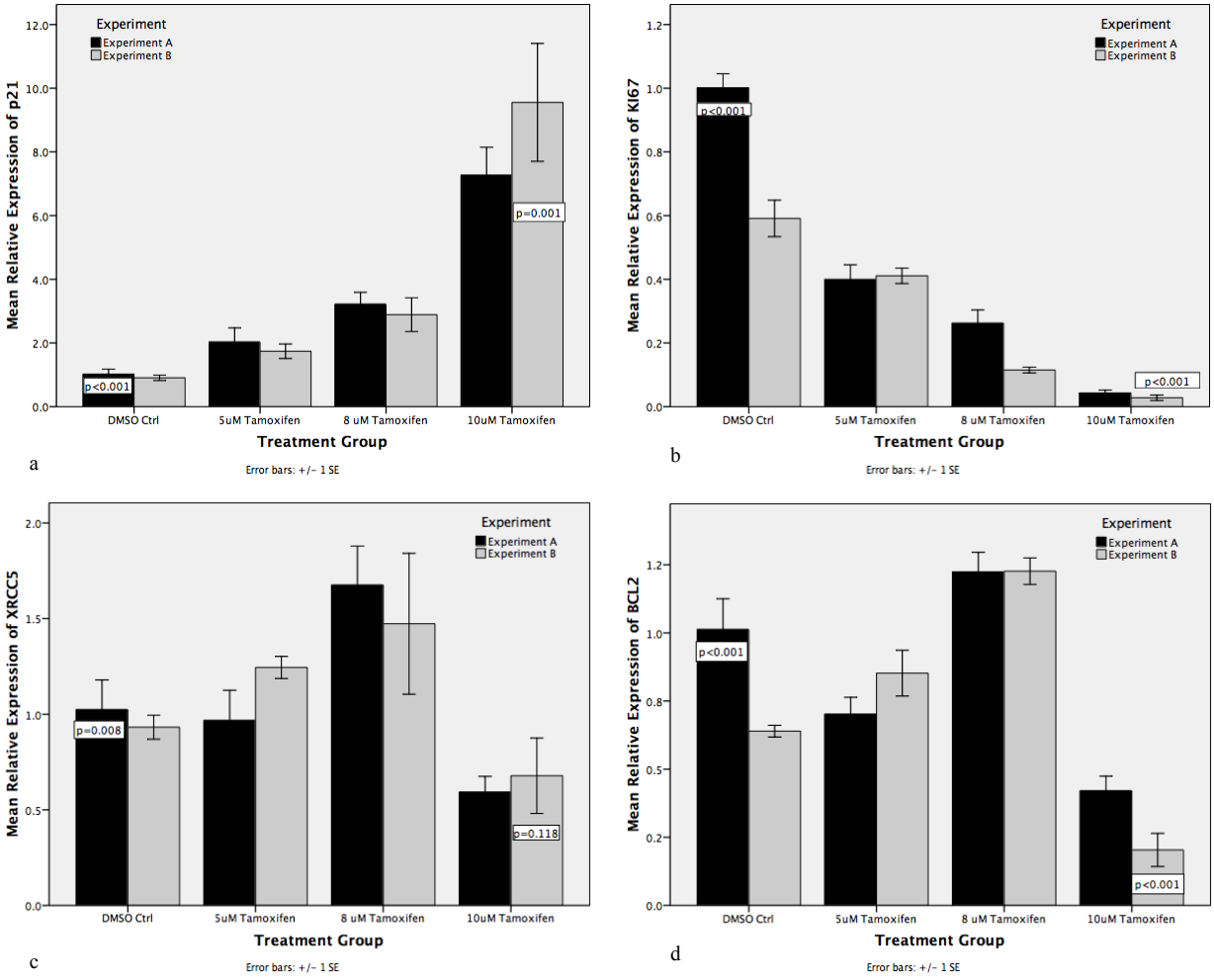

Figure 5.33: Relative transcriptional expression of p21 (a), Ki67 (b), XRCC5 (c) and BCL2 (d) genes in the MDA-MB-453 cell line in response to Tamoxifen treatment.

Only experiment A showed significant changes in the relative transcriptional gene expression of XRCC5 in response to Tamoxifen treatment ($p = 0.008$ for experiment A and $p = 0.118$ for experiment B; Figure 5.33 c). The significant increase the relative transcriptional gene expression of XRCC5 was only observed following treatment with 8μM Tamoxifen (Table 5.3).

In both experiments A and B there were significant changes in the relative gene expression of BCL2 in response to Tamoxifen treatment ($P < 0.001$ for both Experiments; Figure 5.33d). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

5.4.3.2 Combined analysis of duplicate experiments.

Generalized linear model statistical analysis of both duplicate experiments together showed that there was no significant difference between the experiments (p 21, $p = 0.501$; XRCC5, $p = 0.891$; BCL2, $p = 0.106$; Table 5.3). Therefore, the combined data from both experiments can legitimately be analysed.

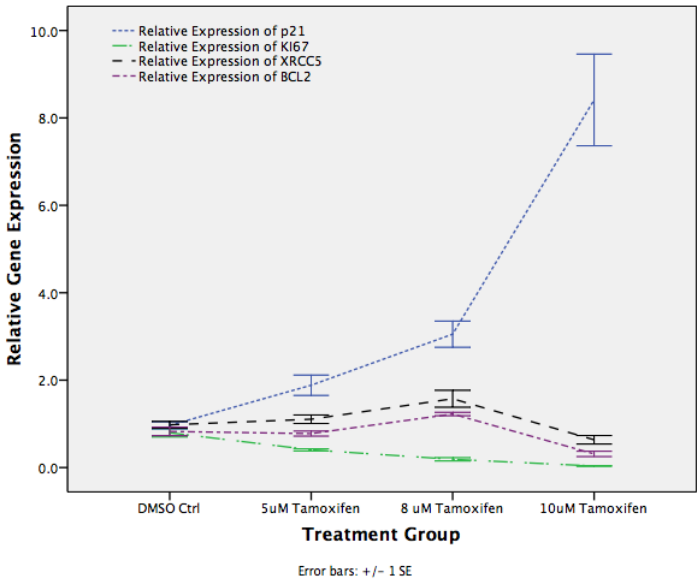

Figure 5.34: Combined data of the duplicate experiments measuring the relative transcriptional expression of p21, Ki67, XRCC5 and BCL2 genes in the MCF-7 cell line in response to Tamoxifen treatment.

The combined analysis showed that there was a significant increase in the relative transcriptional gene expression of p21 after Tamoxifen treatment (Figure 5.34, $p < 0.001$). There was a significant changes in the relative transcriptional gene expression of XRCC5 after Tamoxifen treatment $p = 0.001$; with a significant increase in the relative transcriptional gene expression of XRCC5 at 8μM Tamoxifen concentration (Figure 5.34 and Table 5.3).

The combined analysis of data from both experiments showed a significant decrease in the relative transcriptional gene expression of BCL2 ($p < 0.001$) after treatment with 8μM Tamoxifen (Figure 5.18 and Table 5.2). In spite of the presence of significant experimental difference between the duplicate experiments for KI67, $p = 0.005$), the combined analysis of data from both experiments showed a significant decrease in the relative transcriptional gene expression of KI67 genes in response to increasing concentrations of Tamoxifen ($p < 0.001$, Figure 5.34). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

5.4.4 SIRT1 gene expression in response to Tamoxifen treatment in MDA-MB-453 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT1 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5. 35 shows the relative gene expression of SIRT1 in 5μM and 8μM Tamoxifen treated cells after 72hrs.

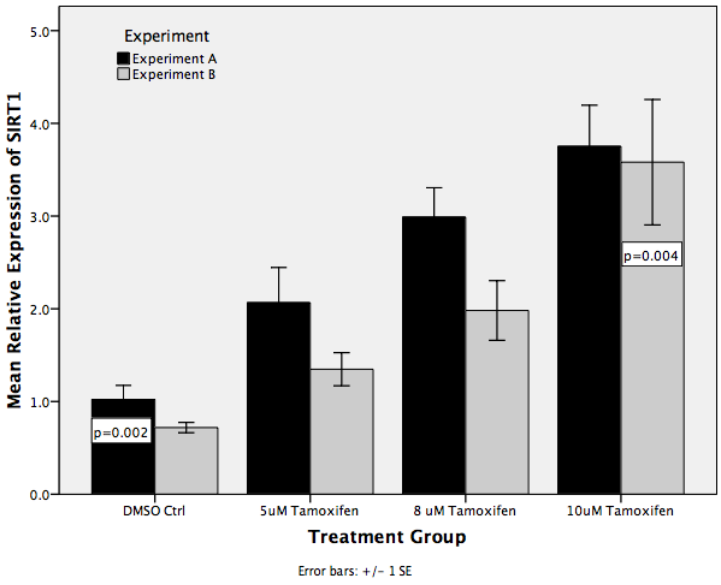

Figure 5.35: Relative transcriptional gene expression of SIRT1 in the MDA-MB-453 cell line in response to Tamoxifen treatment.

This experiment has been performed in duplicate: Exp. A dark column, and Exp. B light column.

The data shows that there was a significant increase in the relative transcriptional gene expression of SIRT1 in response to increasing concentrations of Tamoxifen in the MDA-MB-453 cells ($p = 0.002$ for experiment A and $p = 0.004$ for experiment B, Figure 5.35). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

Although the generalized linear model statistical analysis showed a significant difference between the two duplicate experiments that there was no significant difference between the experiments ($p = 0.039$, Figure 5.37), similar significant increases were observed for relative transcriptional gene expression of SIRT1 in response to increasing concentrations of Tamoxifen when both experiments were combined and analysed together ($P < 0.001$, Figure 5.37). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

5.4.5 SIRT2 gene expression in response to Tamoxifen treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the relative transcriptional gene expression of SIRT2 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.36 shows the relative gene expression of SIRT2 in MDA-MB-453 cells treated with Tamoxifen for 72 hrs.

The data showed that there was a significant increase in the relative transcriptional gene expression of SIRT2 in response to increasing concentrations of Tamoxifen in the MDA-MB-453 cells ($p < 0.001$ for both experiment A and B, Figure 5.36). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

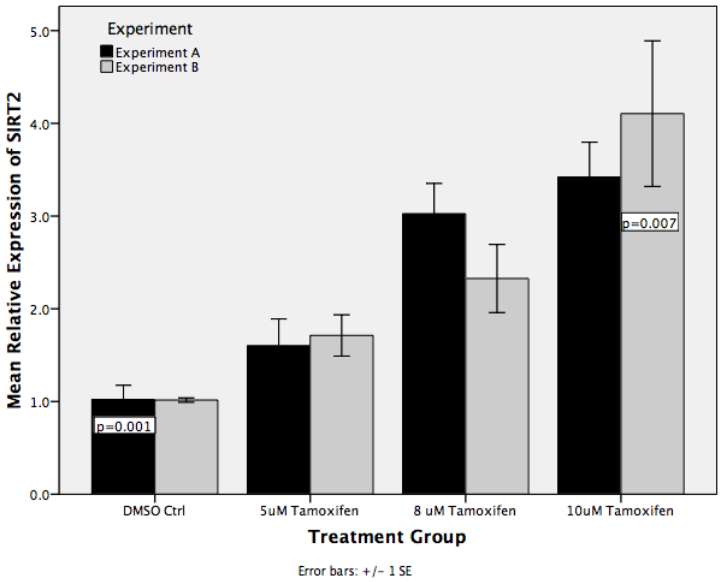

Figure 5.36: Relative transcriptional gene expression of SIRT2 in the MDA-MB-453 cell line in response to Tamoxifen treatment.

The generalized linear model statistical analysis proved that there was no significant experimental difference between the duplicate experiments ($p = 0.932$, Table 5.3). The

analysis of the combined data showed that there was a significant increase in the relative transcriptional gene expression of SIRT2 following Tamoxifen treatment ($p < 0.001$, Figure 5.37, Table 5.3).

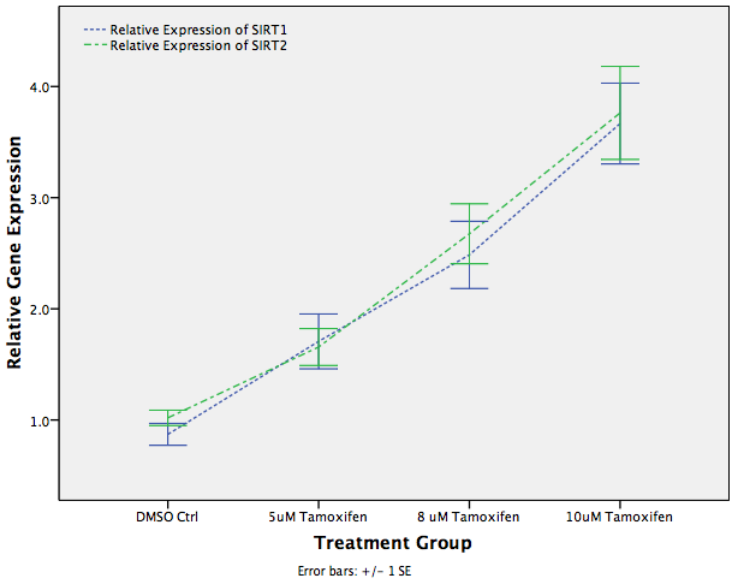

Figure 5.37: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT1 and SIRT2 genes in the MDA-MB-453 cell line in response to Tamoxifen treatment.

5.4.6 SIRT3, SIRT4 & SIRT5 gene expression in response to Tamoxifen treatment in MDA-MB-231 cells.

SIRT3, SIRT4 & SIRT5 are known to be mitochondrial related sirtuins and all show responses to Tamoxifen treatment. Therefore they are presented together in this section. Real time PCR was used to quantify the transcriptional expression of SIRT3, SIRT4 and SIRT5 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.38 a, b, and c shows the relative gene expression of SIRT3, SIRT4 and SIRT5 in 5μM and 8μM Tamoxifen treated cells after 72hrs.

5.4.6.1 Separate analysis of duplicate experiments.

Experiment A showed that there were significant overall changes in the relative transcriptional levels of SIRT3, SIRT4 and SIRT5 genes in response to Tamoxifen treatment ($p < 0.001$, $p = 0.004$ and $p = 0.045$ respectively; Figure 5.38 a, b, and c). However, in experiment B overall significant changes were observed for SIRT3 $p = 0.054$ and SIRT4 $p = 0.003$ but not for SIRT5 $p = 0.179$ (Figure 5.38 a, b, and c). It is also clear

from Figure 5.35 that the changes in SIRT4 gene expression are better correlated with increasing concentration of Tamoxifen than the changes in SIRT3 and SIRT5 gene expression. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

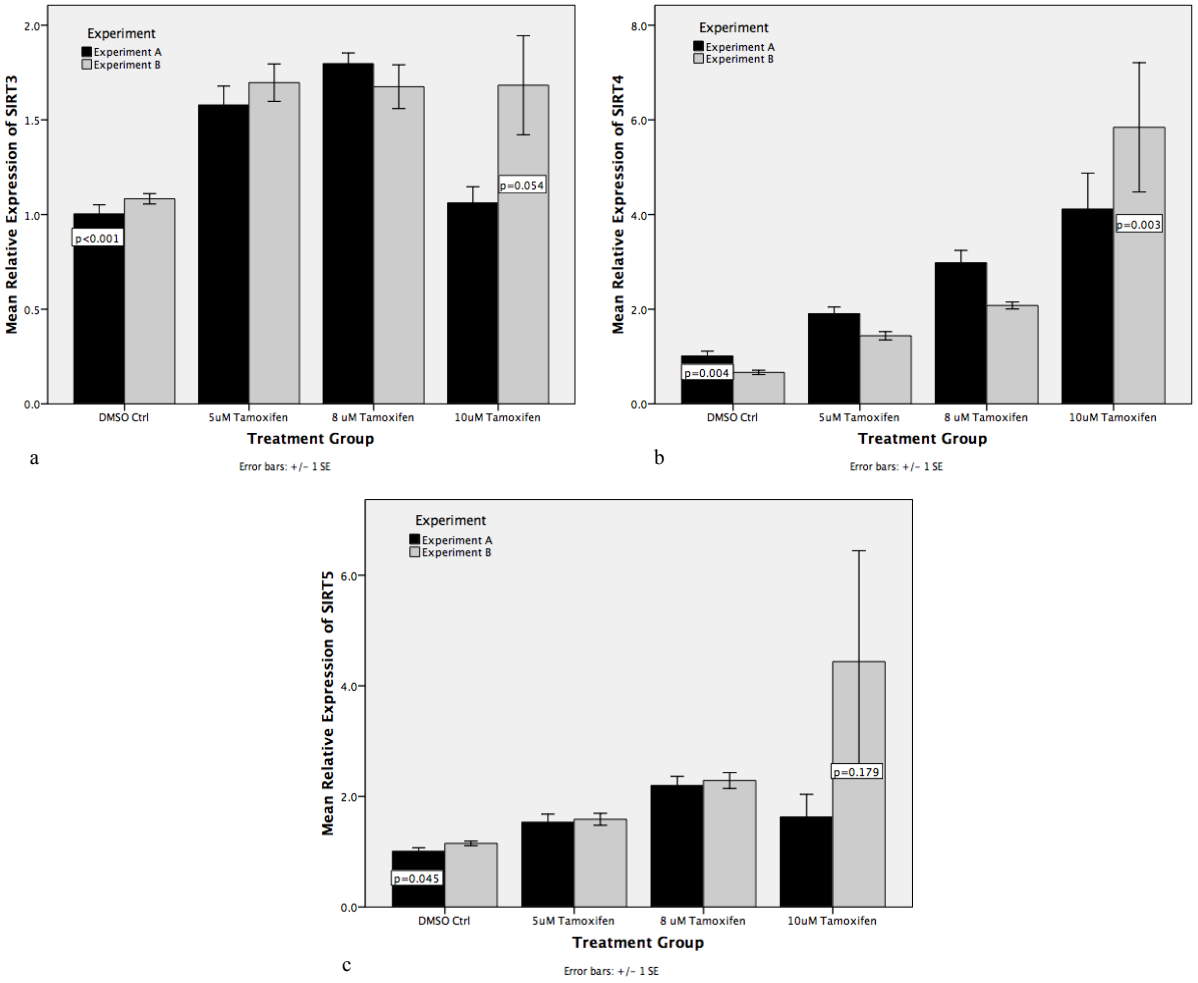

Figure 5.38: Relative transcriptional expression of SIRT3 (a), SIRT4 (b), and SIRT5 (c) genes in the MDA-MB-453 cell line in response to Tamoxifen treatment.

5.4.6.2 Combined analysis of duplicate experiments.

Generalized linear model statistical analysis of both duplicate experiments together showed that there was no significant difference between the experiments for all three genes (SIRT3, $p = 0.096$; SIRT4, $p = 0.991$; SIRT5, $p = 0.171$; Table 5.3). Therefore, the combined data from both experiments can legitimately be analysed.

The combined analysis for both experiments showed significant changes in the relative transcriptional expression of SIRT3 ($p < 0.001$), and SIRT4 ($p < 0.001$) after Tamoxifen

treatment (Figure 5.39). Conversely, changes in the relative transcriptional expression of SIRT5 gene after Tamoxifen treatment were not significant (p = 0.098, Figure 5.39). The significant increase in the relative transcriptional expression of SIRT3 was observed only at the 8μM Tamoxifen concentration. Whereas, the significant increase in the relative transcriptional expression of SIRT4 was observed at the 8μM and 10μM Tamoxifen concentration. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

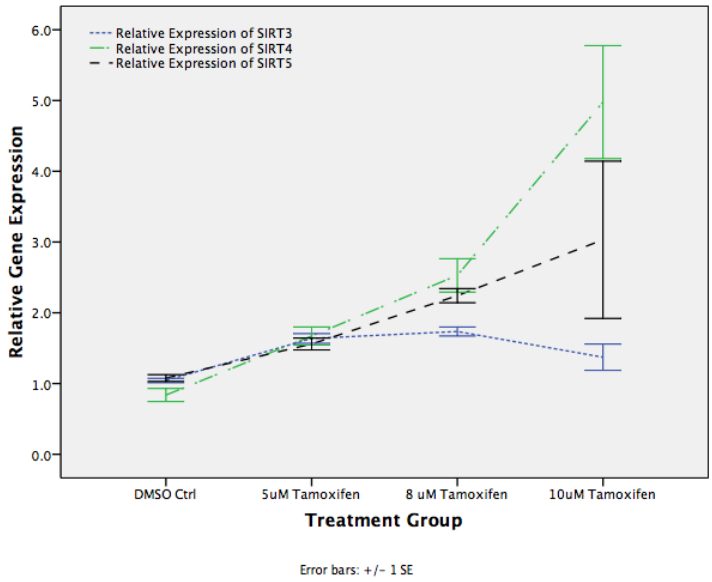

Figure 5.39: Combined data of the duplicate experiments measuring relative transcriptional gene expression of SIRT3, SIRT4 and SIRT5 in the MDA-MB-453 cell line in response to Tamoxifen treatment.

5.4.7 SIRT6 gene expression in response to Tamoxifen treatment in MDA-MB-453 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT6 in relation to HPRT a housekeeping gene as described in the Material and Methods. Figure 5.40 shows the relative gene expression of SIRT6 in MDA-MB-453 cells treated with Tamoxifen for 72 hrs.

Experiment A showed no significant changes for relative gene expression of SIRT6 in the MDA-MB-453 cell line when treated with Tamoxifen for 72 hours ($p < 0.113$ Figure 5.40). However, there were significant changes in the relative levels of SIRT6 gene expression following Tamoxifen treatment in experiment B (p = 0.037; Figure 5.37). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

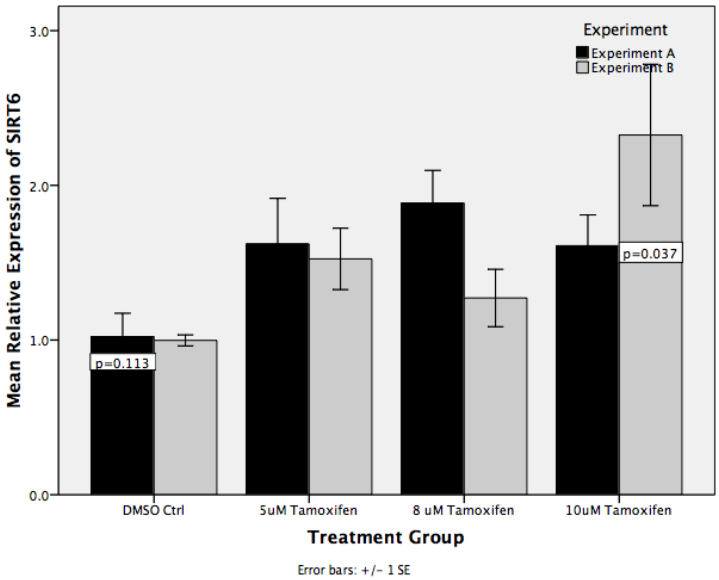

Figure 5.40: Relative transcriptional gene expression of SIRT6 in the MDA-MB-453 cell line in response to Tamoxifen treatment.

The generalized linear model statistical analysis indicated that there was no significant difference between the experiments ($p = 0.989$, Table 5.3). Therefore, both experiments could be combined and analysed together.

The combined analysis for both experiments showed significant changes in the relative transcriptional expression of SIRT6 after Tamoxifen treatment ($P < 0.019$, Figure 5.42). The significant increase in the relative transcriptional expression levels of SIRT6 gene was mainly observed at 10 μM Tamoxifen concentration. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

5.4.8 SIRT7 gene expression in response to Tamoxifen treatment in MDA-MB-231 cells.

Real time PCR was used to quantify the transcriptional expression of SIRT7 in relation to HPRT as a housekeeping gene as described in the Material and Methods. Figure 5.41 shows the relative gene expression of SIRT7 in Tamoxifen treated cells after 72hrs.

Both duplicate experiments showed that relative transcriptional gene expression of SIRT7 increased significantly in the MDA-MB-453 cells when treated with Tamoxifen for 72 hrs ($p = 0.039$ and $p = 0.022$ for experiment A and B respectively, Figure 5.41). The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

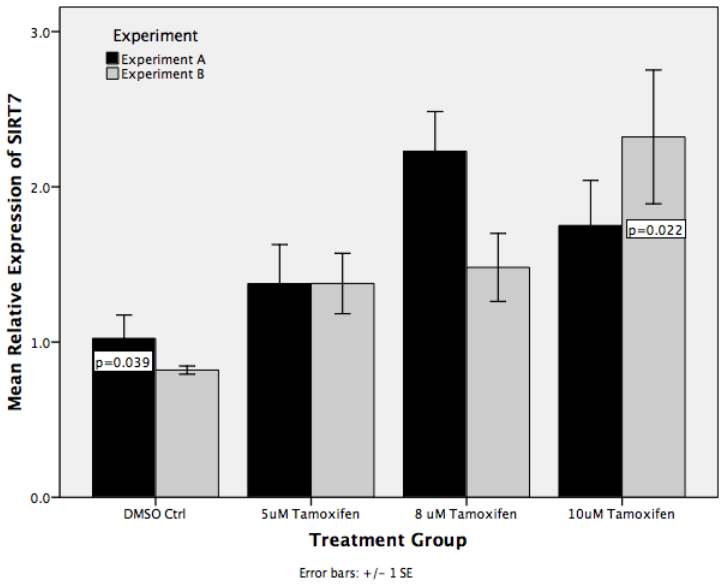

Figure 5.41: Relative transcriptional gene expression of SIRT7 in the MDA-MB-453 cell line in response to Tamoxifen treatment.

The generalized linear model statistical analysis indicated that there was no significant difference between the experiments ($p = 0.636$, Table 5.3). Therefore, both experiments could be combined and analysed together. The combined analysis for both experiments showed significant changes in the relative transcriptional expression of SIRT7 after Tamoxifen treatment ($P < 0.003$, Figure 5.42). The significant increase in the relative transcriptional expression levels of SIRT7 gene was observed at 8μM and 10 μM Tamoxifen concentration. The p value for Dunnett t-test analysis for each treatment is shown in Table 5.3.

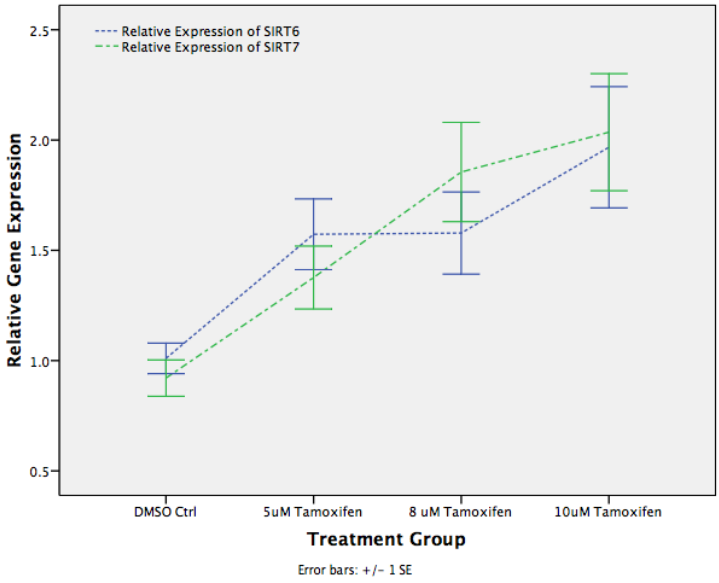

Figure 5.42: Combined data of the duplicate experiments measuring the relative transcriptional expression of SIRT6 and SIRT7 genes in the MDA-MB-453 cell line in response to Tamoxifen treatment.

Table 5.3: Probability levels for Tamoxifen treatment experiments in the MDA-MB-453 cell line.

ANOVA test was used to determine the overall probability of gene expression changes as a result of Tamoxifen treatment. The probability of deviation of each treatment (concentration of drug) from the control (DMSO only) was investigated using the Dunnett t-test. The statistical analysis was performed for each experiment separately and when the data form both experiments were combined together.

Experiment A			Experiment B		Combined Experiments		
P21			**P21**		**P21**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p = 0.446	p = 0.001	p = 0.876	p < 0.001	p = 0.537	p = 0.501
8μM Tamoxifen		p = 0.048		p = 0.39		p = 0.045	
10μM Tamoxifen		p < 0.001		p = 0.001		p < 0.001	
Ki67			**Ki67**		**Ki67**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p < 0.001	p < 0.001	p = 0.01	p < 0.001	p < 0.001	p = 0.005
8μM Tamoxifen		p < 0.001		p < 0.001		p < 0.001	
10μM Tamoxifen		p < 0.001		p < 0.001		p < 0.001	
XRCC5			**XRCC5**		**XRCC5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.008	p = 0.988	p = 0.118	p = 0.621	p = 0.001	p = 0.822	p = 0.891
8μM Tamoxifen		p = 0.045		p = 0.244		p = 0.010	
10μM Tamoxifen		p = 0.197		p = 0.742		p = 0.176	
BCL2			**BCL2**		**BCL2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p = 0.056	p < 0.001	p = 0.078	p < 0.001	p = 0.908	p = 0.106
8μM Tamoxifen		p = 0.209		p < 0.001		p = 0.001	
10μM Tamoxifen		p = 0.002		p = 0.002		p < 0.001	
SIRT1			**SIRT1**		**SIRT1**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.002	0.144	p = 0.004	p = 0.549	p < 0.001	p = 0.07	p = 0.039
8μM Tamoxifen		0.009		p = 0.115		p = 0.001	
10μM Tamoxifen		0.001		p = 0.002		p < 0.001	
SIRT2			**SIRT2**		**SIRT2**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.001	p = 0.43	p = 0.007	p = 0.581	p < 0.001	p = 0.259	p = 0.932
8μM Tamoxifen		p = 0.004		p = 0.165		p = 0.001	
10μM Tamoxifen		p = 0.001		p = 0.003		p < 0.001	
SIRT3			**SIRT3**		**SIRT3**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p < 0.001	p = 0.002	p = 0.054	p = 0.052	p < 0.001	p = 0.408	p = 0.096
8μM Tamoxifen		p = 0.001		p = 0.06		p < 0.001	
10μM Tamoxifen		p = 0.906		p = 0.057		p = 0.079	
SIRT4			**SIRT4**		**SIRT4**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.004	p = 0.348	p = 0.003	p = 0.769	p < 0.001	p = 0.408	p = 0.991
8μM Tamoxifen		p = 0.024		p = 0.382		p = 0.033	
10μM Tamoxifen		p = 0.002		p = 0.002		p < 0.001	
SIRT5			**SIRT5**		**SIRT5**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.045	p = 0.332	p = 0.179	p = 0.98	p = 0.098	p = 0.868	p = 0.171
8μM Tamoxifen		p = 0.019		p = 0.769		p = 0.325	
10μM Tamoxifen		p = 0.226		p = 0.117		p = 0.052	
SIRT6			**SIRT6**		**SIRT6**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.113	p = 0.205	p = 0.037	p = 0.413	p = 0.019	p = 0.128	p = 0.989
8μM Tamoxifen		p = 0.059		p = 0.811		p = 0.123	
10μM Tamoxifen		p = 0.216		p = 0.02		p = 0.006	
SIRT7			**SIRT7**		**SIRT7**		
	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	ANOVA	Dunnett t-test	Experimental difference
5μM Tamoxifen	p = 0.039	p = 0.627	p = 0.022	p = 0.357	p = 0.003	p = 0.266	p = 0.636
8μM Tamoxifen		p = 0.02		p = 0.245		p = 0.009	
10μM Tamoxifen		p = 0.155		p = 0.009		p = 0.002	

Chapter VI

Discussion 2

Sirtuin gene expression in response to anti-tumour drug treatments

6.1 Sirtuin gene expression in response to Docetaxel treatment in an ER^{-ve} breast cancer cell line (MDA-MB-231).

6.1.1 Docetaxel.

Taxanes are antitumour agents shown to arrest cells in mitosis, through microtubule stabilization and to induce apoptosis. Taxanes stabilize microtubules, enhancing the rate and extent of tubulin polymerization and inhibit depolymerization, through binding to the beta subunit of the tubulin within the microtubule. This results in inhibition of mitotic cell division and cell proliferation, leading to cell death by apoptosis (77, 78). Similarly, the cytotoxic effect of paclitaxel has been correlated both with its ability to interfere with normal microtubule function and with its ability to induce apoptosis (362, 363).

Docetaxel, a semi-synthetic analogue of paclitaxel, is a microtubule-stabilizing taxane, which has recently been approved for use in the clinic for the treatment of breast and prostate cancers and small cell carcinoma of the lung (353). Docetaxel has increased affinity for tubulin (364) and has higher antitumour activity compared with paclitaxel (365).

There is no consensus about the main effect of Docetaxel. Initially Docetaxel was shown to interrupt the normal mitotic process, through the disruption of microtubules (78). However, despite the clear connection to mitosis, apoptosis has generally been accepted to be the predominant mechanism of cell death in response to taxane chemotherapy (366-

369). Docetaxel activates several apoptotic cellular signals, such as protein kinases and caspases (369, 370) and decreases structural and anti-oxidative gene expression (66, 371). However, other data have indicated that other modes of cell death may also contribute significantly to the overall therapeutic response (372-374). This is supported by the observation that the degree of therapeutic response does not correlate with apoptosis and that anti-apoptotic mutations, or altered expression of genes, such as bcl-2, p21, and p53, are not negative predictors of therapeutic efficacy (375-378). Other forms of death include mitotic catastrophe, (379, 380) treatment-induced senescence and lytic necrosis (381).

Adding to the complexity of Docetaxel action, like paclitaxel (382), Docetaxel provoked striking differences in the cell cycle according to the dose administered (383, 384). At low dose Docetaxel induced aberrant mitosis followed by aneuploidy, whereas at high concentration it induced sustained mitotic arrest followed by mitotic slippage (383, 384). Recently, a dose dependent response to Docetaxel in human breast carcinoma cells has been reported, with no apoptosis triggered at low doses in MCF-7 breast cancer cells (383, 385). Moreover, the Bayet-Robert study showed significant dose-dependent differences in 12 of 30 measured metabolites, suggesting dose-dependent metabolic changes in treated cells (385).

Adding together, the mechanism of action of taxanes is not fully understood. The published data from both *in vitro* and clinical studies indicate significant variation in cellular sensitivity to paclitaxel, suggesting that alternative mechanisms may be responsible for the drug's activity. Although this group of drugs is know as non DNA-damaging agents, it has been reported that Docetaxel can causes DNA damage (386).

The main effects of Docetaxel, especially those could be related to this study have been present above. Given the involvement of sirtuins in the MTR and their role in cancer and given the data of this thesis, showing significant alterations in sirtuin gene expression in breast cancer biopsies, these experiments were performed to investigate if sirtuin gene expression is affected by Docetaxel treatment in an ER^{-VE} cell line.

6.1.2 Conclusions and discussion

The relative transcriptional expression of genes of interest was determined in the MDA-MB-231 (ER^{-ve} breast cancer) cell line after 72 hours of Docetaxel (1nM, 10nM, 100nM) treatment. The treatment conditions (time and concentration) were decided upon following relevant optimization experiments.

The control experiment studying four genes that were expected to give responses in gene expression level after Docetaxel treatment showed:

- Dose dependent increases in the levels of p21 gene expression, indicating cellular stress proportional to increasing Docetaxel concentration, and supporting a cell senescence effect of Docetaxel treatment.

- Significant increases in the levels of XRCC5 gene expression (DNA damage response marker) after Docetaxel treatment, suggesting an accumulation of DNA damage in proportion to the Docetaxel concentration.

- An initial increase in the level of KI67 gene expression (proliferation marker) at 1 nM Docetaxel, followed by a significant decrease at higher concentrations of Docetaxel (10nM and 100nM). These changes might reflect different cellular responses at different concentrations of Docetaxel. The observed increase in Ki67 at low Docetaxel concentrations might indicate a defensive cellular response to overcome the cell cycle suppressive effect of Docetaxel. This increase is in keeping with the initial increase of NF-κB that is observed at low concentrations of Docetaxel and with increased cellular sensitivity to Docetaxel treatment after NF-κB inhibition (387-389).

- An initial increase in the levels of BCL2 (anti-apoptotic marker) gene expression at 1 nM and 10 nM followed by a significant decrease at 100 nM. These results suggest that an apoptotic response is triggered at high concentrations of Docetaxel.

The experiment investigating the relative gene expression of sirtuins in response to Docetaxel treatment in MDA-MB-231 cells showed:

- An increase in the level of SIRT1 gene expression, especially at high levels of Docetaxel treatment. This is in keeping with the pro-apoptotic role for SIRT1 in cancer as observed in the biopsy study. It is also consistent with the data for expression of the control genes BCl2 and Ki67, and with a possible apoptotic response at high concentrations.

- Increases in the levels of SIRT2 gene expression proportional to the increasing concentrations of Docetaxel treatments. This is suggestive of a potential involvement of SIRT2 (microtubule deacetylase) in the mitotic arrest caused by

Docetaxel through its contribution to microtubule dysfunction. Furthermore, this result might suggest that mitotic arrest is present, to varying extents, at all the concentrations of Docetaxel used. This supports mitotic interference as the primary mechanism of action of Docetaxel.

- Increases in the levels of SIRT3, SIRT4, and SIRT5 gene expression in response to Docetaxel treatment. This increase was more significant at higher concentrations of Docetaxel and is in keeping with the pro-apoptotic role for SIRT3 in cancer, as observed in our biopsy study. The increased expression of SIRT3, especially at high concentration of Docetaxel, is in agreement with the BCL2, KI67 and SIRT1 results, suggesting that a higher concentration of Docetaxel is required to induce apoptosis. Similarly, the increased levels of SIRT4 and SIRT5 following Docetaxel treatment could reflect metabolic changes associated with Docetaxel treatment, as suggested by (385). Furthermore, these results might suggest that the potential role of SIRT4 and SIRT5 in tumourigenesis is likely to be anti-cancer in similar way to SIRT3 (pro-apoptotic and anti-cancer).

- Increases in the level of SIRT6 gene expression in response to increasing concentrations of Docetaxel. This result is in keeping with the anticancer role of SIRT6 through its involvement in DNA repair and interaction with NF-κB. This result suggests that there is an increase in SSB DNA damage as result of Docetaxel treatment as well as DSB DNA damage. The presence of DSB DNA damage as was indicated by the increase in XRCC5 gene expression after Docetaxel treatment. Furthermore, this result suggests that suppression of NF-κB-dependent gene expression through increased expression of SIRT6 might be involved in the in mechanistic action of Docetaxel. Previous study showed that inhibition of NF-κB augments Docetaxel-induced apoptosis in different cancer cell lines (387-389).

- Increases in the level of SIRT7 gene expression in response to increasing concentrations of Docetaxel. This result is in keeping with the anti-cancer role of SIRT7 that has been supported by the results of the biopsy studies (Chapter 1. 4. 4. 7). Since increased SIRT7 is able to activate p53- and c-myc-dependent transcription, thus promoting apoptosis, the SIRT7 data are in agreement with our previous findings (BCL2, KI67, SIRT1, SIRT3, SIRT4, SIRT5) suggesting that the apoptotic pathway is induced by high concentrations of Docetaxel.

This study demonstrates changes in levels of sirtuin gene expression in response to Docetaxel treatment in the MDA-MB-231 cell line, and clearly indicates the possible involvement of sirtuins in the cellular effects of Docetaxel, particularly mitotic arrest and apoptosis and possibly in other modes of cell death.

6.2 Sirtuin gene expression in response to Tamoxifen treatment in an ER^{+ve} and ER^{-ve} breast cancer cell lines.

6.2.1 Tamoxifen

Endocrine treatment plays an important role in the management of hormone-dependent breast cancer. The non-steroidal anti-estrogen tamoxifen (TAM), a selective estrogen receptor modulator (SERM), is commonly use in breast cancer chemoprevention and it remains the treatment of choice for most women with hormone receptor-positive, invasive breast carcinoma. However, TAM has limitations in therapy, with resistance to treatment being a frequent outcome (356) and moreover, TAM induces uterine proliferation (agonist actions) increasing the risk of endometrial cancer (390).

The anti-cancer therapeutic efficacy of TAM is thought to derive primarily from its ability to compete with estrogens for binding to the Estrogen receptor (ER). This TAM-bound form of ER (TAM-ER) is unable to effectively activate transcription of genes important for the growth and development of oestrogen-dependent tumours. However several studies suggest that a different mechanism could be involved. TAM can actively induce programmed cell death of cancer cells (391, 392). Other data indicated that there are both ER-dependent and ER-independent mechanisms mediating the apoptotic effects of TAM (360, 361).

TAM can induce apoptosis in a time- and dose-dependent manner by modulating Bcl-2 levels in breast cancer cells, and downregulation of Bcl-2 induced by TAM was not accompanied by alterations in p53 levels (6). At the same time TAM induces transcription factors and genes involved in promoting cell cycle progression, including cyclins E and A2, with kinetics that paralleled that of cells cycling in response to oestrogen. Even though the Tamoxifen treated cells are not able to transit through the cell cycle (393).

TAM treatment also could affect DNA directly through DNA damage and modulation of telomerase activity (357, 394). The mechanism of TAM causing DNA damage is not fully understood, but it is known that TAM can cause DNA damage (DSB) and be genotoxic for normal and cancer cells by free radical generation (357). Furthermore, it has been reported that long-term administration of TAM leads to hepatic tumours in rat liver as a result of the formation of DNA adducts. The formation of DNA adducts results from metabolism of TAM to reactive intermediates which bind irreversibly to hepatic DNA, forming DNA adducts (395).

Other studies reported that TAM is able to induce oxidative stress, followed by mitochondrial dysfunction (358-361, 396).

Although the main effect of TAM is to suppress ER^{+ve} cell proliferation, a positive effect on cell proliferation has been shown for TAM at low concentration (397). TAM, similar to oestrogen but to a lesser extent, is able to stimulate human breast cancer cell proliferation at lower concentration in a biphasic mode (397). Furthermore TAM treatment has been shown to cause a reduction in oestrogen receptors *in vivo* (398).

Only the main effects of TAM, especially those that could be related to this study have been presented. Given the well-known involvement of sirtuins in DNA damage repair, the link between some sirtuins and the mitochondrion (refer to the MTR trinity), and the variety of cellular effects of TAM that involve mitochondria, DNA, and proliferation, this study aimed to investigate the potential involvement of sirtuins in TAM treatment by determining the effect of TAM treatment on sirtuin gene expression in both ER^{+ve} and ER^{-ve} cell lines.

6.2.2 Conclusions and discussion.

6.2.2.1 Sirtuin gene expression in response to Tamoxifen treatment in an ER^{+ve} breast cancer cell line (MCF-7).

In this experiment the relative transcriptional expression of genes of interest was determined in MCF-7 (ER^{+ve} breast cancer) cell line after 72 hours of TAM (5 μM, 8 μM and 10 μM treatment. The treatment conditions (time and concentration) were decided upon following relevant optimization experiments. Only the results of 5μM and 8μM TAM treatment are discussed, as 10μM of Tamoxifen was too cytotoxic and RNA could not be extracted successfully.

The control experiment studying four genes that were expected to give responses in gene expression level after Tamoxifen treatment showed:

- A dose-dependent increase in the level of p21 gene expression, indicating cellular stress proportional to the increasing concentration of TAM.

- Significant decreases in both KI67 and BCL2 gene expression levels at the highest dose of TAM (8μM). This result is consistent with the anti-proliferative effect of TAM and with BCL2 induced apoptosis. No initial increase in cell proliferation was observed with the lower dose of TAM used in this study (5μM).

- No significant changes in the levels of gene expression of the DNA damage marker XRCC5, indicating that, if present at all, any DNA damage due to TAM-treatment (both 5μM and 8μM) in MCF-7 cells was not significant and is unlikely to be responsible for the induced apoptosis.

The experiment investigating the relative gene expression of sirtuins in response to Tamoxifen treatment in MCF-7 cells showed:

- Increases in the level of SIRT1 gene expression, especially at high TAM concentration. This result is consistent with the pro-apoptotic role for SIRT1 in cancer as observed in our biopsy study. The data for the control genes BCl2 and Ki67, and with TAM-induced apoptosis. Furthermore, the changes in SIRT1 expression might correlate with its contribution to the loss of ERα that has been noticed after modulating SIRT1 activity (400), or with its role in deacetylating nuclear receptors including the ERα (328).

- Increases in the level of SIRT2 gene expression proportional to increasing concentrations of TAM. This is suggestive of a potential involvement of SIRT2 in the mechanism of action of TAM, explaining the inability of a TAM-treated cell to proceed through the cell cycle in spite of the increases in transcription factors and genes involved in promoting the cell cycle that are associated with TAM treatment (393).

- Significant increases, especially at high TAM concentration (8μM), in the levels of SIRT3, SIRT4, and SIRT5 gene expression in response to TAM treatment. These changes are in keeping with the pro-apoptotic role of SIRT3, and indicate, together

with the BCL2, KI67 and SIRT1 results, that the apoptotic response is associated mainly with the higher concentration of TAM. The increased levels of SIRT4 and SIRT5 are suggestive of a potential involvement for these genes in TAM treatment. This could be attributed to their involvement in metabolism or to a potential role in the cancer disease process, which is more likely to be similar to that of SIRT3 (pro-apoptotic or anti-cancer).

- Significant increases, especially at high TAM concentration (8μM), in the level of SIRT6 gene expression in response to TAM treatment. This result is in keeping with the anticancer role of SIRT6 through its involvement in DNA repair and interaction with NF-κB. The observed increase in SIRT6 gene expression might reflect its involvement in maintaining telomeric stability, that could be affected by TAM (394). Furthermore, these results might suggest that single strand breaks DNA damage could be caused by TAM treatment.

- Significant increases, especially at high concentration (8μM), in the level of SIRT7 gene expression after TAM treatment.

6.2.2.2 Sirtuin gene expression in response to Tamoxifen treatment in an ER^{-ve} breast cancer cell line (MDA-MB-453).

In this experiment the relative transcriptional expression of genes of interest was determined in the MDA-MB-453 (ER^{-ve} breast cancer) cell line after 72 hours of TAM (5μM, 8μM and 10μM treatment. The treatment conditions (time and concentration) were decided upon following relevant optimization experiments.

The changes in the relative transcriptional expression of genes of interest (control genes and sirtuins) in response to TAM treatment in MDA-MB 453 were very similar to those observed in the MCF-7 cell. Therefore the main differences will be discussed below.

- Experimentally a major difference was the ability to extract RNA from MDA-MB-453 treated with all the three chosen concentrations of TAM (5μM, 8μM and 10μM), indicating less sensitivity of the cells to TAM.

- The changes in the levels of XRCC5 were more obvious in MDA-MB-453 cells and showed non-constant significant increase at 8μM TAM (only experiment A). This result is consistent with the DNA damage component of TAM treatment.

- All sirtuin genes showed similar changes in their relative transcriptional levels after TAM treatment in MDA-MB 453 when compared to the MCF-7. However, greater changes in the gene expression of SIRT3 and SIRT4 were observed, suggesting that SIRT4 is more informative in this cell line (MDA-MB-453).

This study demonstrates changes in levels of sirtuin gene expression in response to TAM treatment in both MCF-7 and MDA-MB-453 cell lines. There was no significant difference in the behaviour of sirtuin gene expression in response to TAM treatment between these two cell lines to indicate that sirtuin changes were ER-dependent. These changes indicate possible involvement of sirtuins in the cellular effects of TAM. In particular, the evidence presented for an involvement of SIRT2 in the mechanism of TAM treatment is a novel observation.

The data obtained from these three experiments demonstrate clear changes in levels of sirtuin gene expression in response to Docetaxel treatment in the MDA-MB-231 cell line and in response to TAM treatment in both MCF-7 and MDA-MB-453 cell lines. These changes support the second part of the hypothesis underpinning this thesis: “sirtuins might contribute both to the pathogenesis of cancer and to the response of tissue to anti-tumour agents.”

Therefore, altering the activity of sirtuins with chemical agonists or by the induction of increased gene expression might increase cell sensitivity to both TAM and Docetaxel activity. The data presented in this study point the way to further experiments to investigate this potential synergistic effect of sirtuins on anticancer agents, in which the effects of TAM and Docetaxel on cell lines when the levels of one or more sirtuins are increased, reduced or eliminated are investigated.

Chapter VII

Final Conclusions

7.1 Final conclusions.

The experimental evidence accumulated in this thesis provides an insight into the involvement of sirtuins in breast cancer disease. The decreased levels of the relative transcriptional expression of sirtuin genes in breast cancer biopsies, is consistent with the pro-apoptotic (anti-tumourgenic) effect of sirtuins. The pro- apoptotic role was further supported after the significant increased in sirtuins relative transcriptional expression observed in response to anti-cancer treatments namely: Tamoxifen and Docetaxel.

7.1.1 In vivo *study*

This study compared the relative transcriptional expression levels of the sirtuin genes between normal, non-malignant and malignant breast biopsies. This particular experiment showed a decrease in sirtuin gene expression in breast cancer biopsies and supports an anti-tumourgenic effect role for sirtuins. Decreased SIRT2 and SIRT6 in breast cancer biopsies, when compared to both normal and non-malignant biopsies, plus the association between higher tumour grades and lower levels of relative expression of both SIRT2 and SIRT6, gives insight into how DNA damaged cells could escape the mitotic check points.

The decreased levels of SIRT1 and SIRT6 in breast cancer biopsies and their involvements in negatively regulating the NF-κB cell proliferating effect at two different levels, are worthy of note. SIRT1 can deacetylate and inactivate NF-κB, whereas SIRT6 inhibits the expression of NF-κB target genes through its interaction with the NF-κB RELA subunit. This result might indicate a potential role for NF-κB in breast cancer tumourigenesis that might be promoted thorough decreased levels of SIRT1 and SIRT6.

The lower levels of the mitochondrial sirtuins (SIRT3, SIRT4 and SIRT5) in malignant biopsies are suggestive of potential involvement in breast cancer disease, possibly through interfering with the mitochondrial apoptotic pathway. Finally, lower levels of SIRT7 were an interesting finding as it supports the recently discovered pro-apoptotic effect and contradicting the previously known proliferative effect for SIRT7.

The associations between SIRT1, SIRT6, SIRT7 and patient's outcomes suggest their potential use as additional biological prognostic markers in breast cancer disease. The levels of SIRT6 expression was found to be independent of known prognostic markers in predicting survival and remained significantly associated with survival in breast cancer patients selected to have moderate prognosis. Therefore, it might help in predicting

survival in individuals who have equivocal prognostic pathological markers and provide an explanation for those breast cancer cases that behave unexpectedly, according to the known pathological prognostic markers.

7.1.2 In vitro study.

This study investigated the changes in relative transcriptional expression levels of sirtuin genes in response to Tamoxifen and Docetaxel in breast caner cell lines. Changes in relative transcriptional expression levels of p21, Ki67, XRCC5 and BCL2 were determined first as a control for used treatments. Both Tamoxifen and Docetaxel caused significant increases in the relative transcriptional expression levels of sirtuin genes, supporting the pro-apoptotic effect of sirtuins.

MDA-MB-231 cells treated with Docetaxel for 72 hours showed significant increase in the levels of SIRT2 and suggests that SIRT2 might be implicated in mitotic arrest caused by Docetaxel, possibly through its microtubule deacetylation effect that contributes to microtubule dysfunction. Similarly, an increase in the level of SIRT2 was observed after Tamoxifen treatment in both MCF-7 and MDA-MB-453 cell lines. This increase might suggest an involvement of SIRT2 in preventing TAM-treated cell from proceeding through the cell cycle in spite of increased levels of transcription factors, that promote cell cycle progression, observed after Tamoxifen treatment (393).

Increased levels of SIRT6, XRCC5 is indicative of DNA damage that is associated with higher concentration of both Tamoxifen and Docetaxel. A pro-apoptotic role for SIRT1, SIRT3 and SIRT7, and speculated pro-apoptotic role for the mitochondrial SIRT4 and SIRT5 was indicated by their increased expression in response to both Tamoxifen and Docetaxel.

There was no significant difference in sirtuin changes after Tamoxifen treatment between the MCF-7 (ER^{+ve}) and MDA-MB-453 (ER^{-ve}) cell lines to indicate that sirtuin changes were ER-dependent. However MDA-MB-453 showed lower sensitivity to Tamoxifen treatment.

These results imply that sirtuins are implicated in breast cancer disease, suggesting the possible use of SIRT6 as a novel biological prognostic marker and specify sirtuin activators, rather than inhibitors, might be beneficial in breast cancer disease and enhance the response to adjuvant chemotherapy.

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Appendices

Appendix 1

Primers and probe sequence of sirtuins and their position on the messenger RNA (mRNA) sequence. Black squares represent the exon exom boundaries. The sequence position for primers and probe and highlighted with different colours

- Forward with Green
- Reverse with Blue
- Probe with red

Only mRNA sequence was presented for SIRT4, SIRT5, SIRT6 since the primers and probes sequence for these sirtuins are not provided form the supplying company (Applied Biosystems).

Homo sapiens SIRT1

ACCESSION NM_012238
VERSION NM_012238.3 GI:13775598

1 GTCGAGCGGG AGCAGAGGAG GCGAGGGAGG AGGGCCAGAG AGGCAGTTGG AAGATGGCGG
61 ACGAGGCGGC CCTCGCCCTT CAGCCCGGCG GCTCCCCCTC GGCGGCGGGG GCCGACAGGG
121 AGGCCGCGTC GTCCCCCGCC GGGGAGCCGC TCCGCAAGAG GCCGCGGAGA GATGGTCCCG
181 GCCTCGAGCG GAGCCCGGGC GAGCCCGGTG GGGCGGCCCC AGAGCGTGAG GTGCCGGCGG
241 CGGCCAGGGG CTGCCCGGGT GCGGCGGCGG CGGCGCTGTG GCGGGAGGCG GAGGCAGAGG
301 CGGCGGCGGC AGGCGGGGAG CAAGAGGCCC AGGCGACTGC GGCGGCTGGG GAAGGAGACA
361 ATGGGCCGGG CCTGCAGGGC CCATCTCGGG AGCCACCGCT GGCCGACAAC TTGTACGACG
421 AAGACGACGA CGACGAGGGC GAGGAGGAGG AAGAGGCGGC GGCGGCGGCG ATTGGGTACC
481 GAG[redacted]ATAACCT TCTGTTCGGT GATGAAATTA TCACTAATGG TTTTCATTCC TGTGAAAGTG
541 ATGAGGAGGA TAGAGCCTCA CATGCAAGCT CTAGTGACTG GACTCCAAGG CCACGGATAG[redacted]
601 GTCCATATAC TTTTGTTCAG CAACATCTTA TGATTGGCAC AGATCCTCGA ACAATTCTTA
661 AAGATTTATT GCCGGAAACA ATACCTCCAC CTGAGTTGGA TGATATGACA CTGTGGCAGA
721 TTGTTATTAA TATCCTTTCA GAACCACCAA AAAGGAAAAA AAGAAAAGAT ATTAATACAA
781 TTGAAGATGC TGTGAAATTA CTGCAAGAGT GCAAAAAAAT TATAGTTCTA ACTGGAGCTG
841 GG[redacted]GTGTCTGT TTCATGTGGA ATACCTGACT TCAGGTCAAG GGATGGTATT TATGCTCGCC
901 TTGCTGTAGA CTTCCCAGAT CTTCCAGATC CTCAAGCGAT GTTTGATATT GAATATTTCA
961 GAAAAGATCC AAGACCATTC TTCAAGTTTG CAAAG[redacted]GAAAT ATATCCTGGA CAATTCCAGC

1021 CATCTCTCTG TCACAAATTC ATAGCCTTGT CAGATAAGGA AGGAAAACTA CTTCGCAACT
1081 ATACCCAGAA CATAGACACG CTGGAACAGG TTGCGGGAAT CCAAAGGATA ATTCAGTGTC
1141 ATG[redacted]GTTCCTT TGCAACAGCA TCTTGCCTGA TTTGTAAATA CAAAGTTGAC TGTGAAGCTG
1201 TACGAGGAGA TATTTTTAAT CAG[redacted]GTAGTTC CTCGATGTCC TAGGTGCCCA GCTGATGAAC
1261 CGCTTGCTAT CATGAAACCA GAGATTGTGT TTTTTGGTGA AAATTTACCA GAACAGTTTC
1321 ATAGAGCCAT GAAGTATGAC AAAGATGAAG TTGACCTCCT CATTGTTATT GGGTCTTCCC
1381 TCAAAGTAAG ACCAGTAGCA CTAATTCCAA [redacted]GTTCCATACC CCATGAAGTG CCTCAGATAT
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2641 TTAAAGTTTT CAAAAAGCCA TCGGAATGTT AAATTAATGT AAAGGGACAG CTAATCTAGA
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2881 ACAATATGTG GGGAGAGCAC TCGGTTGTCT TTACTTTTAA AAGTAATACT TGGTGCTAAG
2941 AATTTCAGGA TTATTGTATT TACGTTCAAA TGAAGATGGC TTTTGTACTT CCTGTGGACA
3001 TGTAGTAATG TCTATATTGG CTCATAAAAC TAACCTGAAA AACAAATAAA TGCTTTGGAA
3061 ATGTTTCAGT TGCTTTAGAA ACATTAGTGC CTGCCTGGAT CCCCTTAGTT TTGAAATATT
3121 TGCCATTGTT GTTTAAATAC CTATCACTGT GGTAGAGCTT GCATTGATCT TTTCCACAAG
3181 TATTAAACTG CCAAAATGTG AATATGCAAA GCCTTTCTGA ATCTATAATA ATGGTACTTC
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3301 CCCTGATTAT ACAGTTCCAA AGTAATAAGA TGTTAATTGT AATTCAGCCA GAAAGTACAT
3361 GTCTCCCATT GGGAGGATTT GGTGTTAAAT ACCAAACTGC TAGCCCTAGT ATTATGGAGA
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4021 AACCAGTGTT TTTTACTTGT ACACTGTTTT AAAGTCTATT AAAATTGTCA TTTGACTTTT
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Homo sapiens SIRT2

ACCESSION NM_012237
VERSION NM_012237.2 GI:13775599

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121 AGCAGTCGGT GACGGGACAC AGTGGTTGGT GACGGGACAG AGCGGTCGGT GACAGCCTCA
181 AGGGCTTCAG CACCGCGCCC ATGGCAGAGC CAGACC[redacted]CCTC TCACCCTCTG GAGACCCAGG
241 CAGGGAAGGT GCAGGAGGCT CAG[redacted]GACTCAG ATTCAGACTC TGAGGGAGGA GCCGCTGGTG
301 GAGAAGCAGA CA[redacted]TGGACTTC CTGCGGAACT TATTCTCCCA GACGCTCAGC CTGGGCAGCC
361 AGAAGGAGCG TCTGCTGGAC GAGCTGACCT TGGAAGGGGT GGCCCGGTAC ATGCAGAGCG
421 AACGCT[redacted]GTCG CAGAGTCATC TGTTTGGTGG GAGCTGGAAT CTCCACA[redacted]TCC GCAGGCATCC
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Homo sapiens SIRT3

ACCESSION AF083108
VERSION AF083108.2 GI:13259626

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181 GGCGTGGGGC CGTTTCAGGC CTGCGGCTGT CGGCTGGTGC TTGGCGGCAG GGACGATGTG
241 AGTGCGGGGC TGAGAGGCAG CCATGGGGCC CGCGGTGAGC CCTTGGACCC GGCGCGCCCC
301 TTGCAGAGGC CTCCCAGACC CGAGGTGCCC AGGGCATTCC GGAGGCAGCC GAGGGCAGCA
361 GCTCCCAGTT TCTTCTTTTC GAG[redacted]TATTAAA GGTGGAAGAA GGTCCATATC TTTTTCTGTG
421 GGTGCTTCAA GTGTTGTTGG AAGTGGAGGC AGCAGTGACA AGGGGAAGCT TTCCCTGCAG
481 GATGTAGCTG AGCTGATTCG GGCCAGAGCC TGCCAGAGGG TGGTGGTCAT GGTGGGGGCC
541 GGCATCAGCA CACCCAGTGG CATTCCAGAC TTCAG[redacted]ATCGC CGGGGAGTGG CCTGTACAGC
601 AACCTCCAGC AGTACGATCT CCCGTACCCC GAGGCCATTT TTGAACTCCC ATTCTTCTTT
661 CACAACCCCA AGCCCTTTTT CACTTTGGCC AAGGAGCTGT ACCCTGGAAA CTACAAGCCC
721 AACGTCACTC ACTACTTTCT CCGGCTGCTT CATGACAAGG GGCTGCTTCT GCGGCTCTAC
781 ACGCAGAACA TCGATGGGCT TGAGAGAG[redacted]TG TCGGGCATCC CTGCCTCAAA GCTGGTTGAA
841 GCTCATGGAA CCTTTGCCTC TGCCACCTGC ACAGTCTGCC AAAGACCCTT CCCAGGGGAG
901 GACATTCGG[redacted]G CTGACGTGAT GGCAGACAGG GTTCCCCGCT GCCCGGTCTG CACCGGCGTT
961 GTGAAGCCCG ACATTGTGTT CTTTGGGGAG CCGCTGCCCC AGAGGTTCTT GCTGCATGTG
1021 GTTGATTTCC CCATGGCAGA TCTGCTGCTC ATCCTTGGGA CCTCCCTGGA G[redacted]GTGGAGCCT
1081 TTTGCCAGCT TGACCGAGGC CGTGCGGAGC TCAGTTCCCC GACTGCTCAT CAACCGGGAC
1141 TTGGTGGGGC CCTTGGCTTG GCATCCTCGC AGCAGGGACG TGGCCCAGCT GGGGGACGTG
1201 GTTCACGGCG TGGAAAGCCT AGTGGAGCTT CTGGGCTGGA CAGAAGAGAT GCGGGACCTT
1261 GTGCAGCGGG AAACTGGGAA G[redacted]CTTGATGGA CCAGACAAAT AGGATGATGG CTGCCCCCAC
1321 ACAATAAATG GTAACATAGG AGACATCCAC ATCCCAATTC TGACAAGACC TCATGCCTGA
1381 AGACAGCTTG GGCAGGTGAA ACCAGAATAT GTGAACTGAG TGGACACCCG AGGCTGCCAC
1441 TGGAATGTCT TCTCAGGCCA TGAGCTGCAG TGACTGGTAG GGCTGTGTTT ACAGTCAGGG
1501 CCACCCCGTC ACATATACAA AGGAGCTGCC TGCCTGTTTG CTGTGTTGAA CTCTTCACTC
1561 TGCTGAAGCT CCTAATGGAA AAAGCTTTCT TCTGACTGTG ACCCTCTTGA ACTGAATCAG
1621 ACCAACTGGA ATCCCAGACC GAGTCTGCTT TCTGTGCCTA GTTGAACGGC AAGCTCGGCA
1681 TCTGTTGGTT ACAAGATCCA GACTTGGGCC GAGCGGTCCC CAGCCCTCTT CATGTTCCGA
1741 AGTGTAGTCT TGAGGCCCTG GTGCCGCACT TCTAGCATGT TGGTCTCCTT TAGTGGGGCT
1801 ATTTTTAATG AGAGAAAATC TGTTCTTTCC AGCATGAAAT ACATTTAGTC TCCTCAAAAA
1861 AAAAAAACA

Homo sapiens SIRT4

ACCESSION NM_012240
VERSION NM_012240.1 GI:6912661

1 GTCCGTAGAG CTGTGAGAG[redacted]A ATGAAGATGA GCTTTGCGTT GACTTTCAGG TCAGCAAAAG
61 GCCGTTGGAT CGCAAACCCC AGCCAGCCGT GCTCGAAAGC CTCCATTGGG TTATTTGTGC
121 CAGCAAGTCC TCCTCTGGAC CCTGAGAAGG TCAAAGAGTT ACAGCGCTTC ATCACCCTTT
181 CCAAGAGACT CCTTGTGATG ACTGGGGCAG GAATCTCCAC CGAATCGGGG ATACCAGACT
241 ACAGGTCAGA AAAAGTGGGG CTTTATGCCC GCACTGACCG CAGGCCCATC CAGCATGGTG
301 ATTTTGTCCG GAGTGCCCCA ATCCGCCAGC GGTACTGGGC GAGAAACTTC GTAGGCTGGC
361 CTCAATTCTC CTCCCACCAG CCTAACCCTG CACACTGGGC TTTGAGCACC TGGGAGAAAC
421 TCGGAAAGCT GTACTGGTTG GTGACCCAAA ATGTGGATGC TTTGCACACC AAGGCGGGGA
481 GTCGGCGCCT GACAGAGCTC CACGGATGCA TGGACAG[redacted]GGT CCTGTGCTTG GATTGTGGGG
541 AACAGACTCC CCGGGGGGTG CTGCAAGAGC GTTTCCAAGT CCTGAACCCC ACCTGGAGTG
601 CTGAGGCCCA TGGCCTGGCT CCTGATGGTG ACGTCTTTCT CTCAGAGGAG CAAGTCCGGA
661 GCTTTCAGGT CCCAACCTGC GTTCAATGTG GAGGCCATCT GAAACCAGAT GTCGTTTTCT
721 TCGGGGACAC AGTGAACCCT GACAAGGTTG ATTTTGTGCA CAAGCGTGTA AAAGAAGCCG
781 ACTCCCTCTT GGTGGTGGGA TCATCCTTGC AG[redacted]GTATACTC TGGTTACAGG TTTATCCTCA
841 CTGCCTGGGA GAAGAAGCTC CCGATTGCAA TACTGAACAT TGGGCCCACA CGGTCGGATG
901 ACTTGGCGTG TCTGAAACTG AATTCTCGTT GTGGAGAGTT GCTGCCTTTG ATAGACCCAT
961 GCTGACCACA GCCTGATATT CCAGAACCTG GAACAGGGAC TTTCACTTGA ATCTTGCTGC
1021 TAAATGTAAA TGCCTTCTCA AATGACAGAT TCCAGTTCCC ATTCAACAGA GTAGGGTGCA

1081 CTGACAAAGT ATAGAAGGTT CTAGGTATCT TAATGTGTGG ATATTCTTAA TTAAAACTCA
1141 TTTTTTTTAA ATAAAAAATT GTTCAGCTTT AAAA

Homo sapiens SIRT5

ACCESSION NM_031244
VERSION NM_031244.1 GI:13787214

1 ATTCGGGGGC GCGAGCTGCC CCAG[redacted]TAAATG GAAATGTTTT CTAACATATA AAAACCTACA
61 GAAGAAGAAA ATAATTTTCT GGATCAAATT AGAAGTCTGT ATTATATTGA TGTCTCCAGA
121 TTCAAATATA TTAGAAAGCA GCCGTGGAGA CAACCATCTT CATTTTGGGC GAAATAACTA
181 AAG[redacted]CCCGCCT CAAGCATTAG AACTACAGAC AAACCCTGAT GCGACCTCTC CAGATTGTCC
241 CAAGTCGATT GATTTCCCAG CTATATTGTG GCCTGAAGCC TCCAGCGTCC ACACGAAACC
301 AGATTTGCCT GAAAATGGCT CGGCCAAGTT CAA[redacted]GTATGGC AGATTTTCGA AAGTTTTTTG
361 CAAAAGCAAA GCACATAGTC ATCATCTCAG GAGCTGGTGT TAGTGCAGAA AGTGGTGTTC
421 CGACCTTCAG AGGAGCTGGA GGTTATTGGA GAAAATGGCAAGCCCAG[redacted]GAC CTGGCGACTC
481 CCCTGGCCTT TGCCCACAAC CCGTCCCGGG TGTGGGAGTT CTACCACTAC CGGCGGGAGG
541 TCATGGGGAG CAAGGAGCCC AACGCCGGGC ACCGCGCCAT AGCCGAGTGT GAGACCCGGC
601 TGGGCAAGCA GGGCCGGCGA GTCGTGGTCA TCACCCAGAA CATCGATGAG CTGCACCGCA
661 AGGCTGGCAC CAAGAACCTT CTGGAGATCC ATG[redacted]GTAGCTT ATTTAAAACT CGATGTACCT
721 CTTGTGGAGT TGTGGCTGAG AATTACAAGA GTCCAATTTG TCCAGCTTTA TCAGGAAAAG
781 G[redacted]TGCTCCAGA ACCTGGAACTCAAGATGCCAGCATCCCAGT TGAGAAACTT CCCCG[redacted]GTGTG
841 AAGAGGCAGG CTGCGGGGGC TTGCTGCGAC CTCACGTCGT GTGGTTTGGA GAAAACCTGG
901 ATCCTGCCAT TCTGGAGGAG GTTGACAGAG AGCTCGCCCA CTGTGATTTA TGTCTAGTG[redacted]G
961 TGGGCACTTC CTCTGTGGTG TACCCAGCAG CCATGTTTGC CCCCCAGGTG GCTGCCAGGG
1021 GCGTGCCAGT GGCTGAATTT AACACGGAGA CCACCCCAGC TACGAACAGA TTCAG[redacted]TCATT
1081 TGATCTCCAT CTCATCTCTA ATTATTATAA AGAATTAAAA CAAGTCATCA TTGTAGAAAA
1141 GCAAGAAAAT GCAGATAGAG AAAAAGAAGA AAATAAAACT GGAGTATTTCCACAACCCAA
1201 GTTTAGAGTT GGCCCCCACC TCCCATGCCA TGGACTGAGC AGCAGGGGCC CAGCATCCCT
1261 TGGATATGGT GGCTGTGTCT TCATGTGAAA GAAACTGAAC TTGGTGGTTT TTCCTGCCAG
1321 TTCAGGAGAG ATTCTTGGCA TGTAATATAT ATCACTGCTC AAGTCAAGCC TCCTAAAACC
1381 ACAGACCTGT TTCAGCTGCT ACTTCAGCCA AAATTCTTCA GCTTCATATT GTCTTGAAAA
1441 CCTATGATTG TCTCTAACAA ACAGGCTACT TGCTAGTTAG AAATTCTTAT CAATTTGGCA
1501 AGCTACTTAT CAACCAGACT GACCACAAGA ACTGTCATCT CATCAATGAA GGAGTAACTG
1561 ATCAATGAAG CCAGCAATGC TTTTTTCTTG GCATCATCAA AGCTGACATT TAGAAGAGAT
1621 GCTGGTGATA GTCATCTCAT CCTACTCAAT TTTTCAAAGG CAGAAACCAA CCCTGGAGCA
1681 ATTGAGAGGA CTGTTTAAAC ACAGAGCTTA ACAATGGCAG AATTGTATAT CTCGTGCTTA
1741 ACAGATTTTG GTTGAACTTT ACCCTAGGTC AGGGGTCAGC AAACTACTGC CTGTGGGCCA
1801 AATTTGCCCA CCACCTGTAT CTGTAAATAA GGTTTCATTG GAACACAGCT GTGGCCATAT
1861 GTTTGTATAT TGTGTGTGGC TGCTTTTGCA TTAGGATGAC AGAGGTGAAT AGTTGCAACA
1921 GAGACTGGCT GGTCTGCAAA GCCTAAAATA TGTCCTGTGT GGCCCTTTAC AGAAAAAGTT
1981 TTCTAACCCC TGCTCTAGGT TACGGAGAAA AAAAAATGGA ATAATGTTCT CTGCTACTTT
2041 TAACCTGATT TTCTTTGTAC CTAAATAGGC AGCTAGAATG CTGCCTATAT TTTAATAAGG
2101 ATTTGGATCT CACAAGACAC CTTAGGCCTA CACAAGTTGT TCAGATTCTT TGCCCCAGTT
2161 CTAATCTAGT GACAAAGGCA TAGAATTCTC CTCCCACAGG AATGTATTTC TATTTTCAAG
2221 GTGTTAATTA GTTCCAGTTT TGGTTTTGTC GTTTTCCCCA TGTCCGATGC TTATATTGGA
2281 TGATTTCTGA TAAACCTGAC TATTCCAATA AACCCTAGGC ATTTTTGAAT TTAAAAAAAA
2341 AAAAAAAAAA

Homo sapiens SIRT6

ACCESSION NM_016539
VERSION NM_016539.1 GI:7706709

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   1 GCTTCCGGCG GAAGCGGCCT CAACAAGGGA AACTTTATTG TTCCCGTGGG GCAGTCGAGG
  61 ATGTCGGTGA ATTACGCGGC GGGGCTGTCG CCGTACGCGG ACAAGGGCAA GTGCGGCCTC
 121 CCGG[illegible]AGATCT TCGACCCCCC GGAGGAGCTG GAGCGGAAGG TGTGGGAACT GGCGAGGCTG
 181 GTCTGGCAGT CTTCCAGTGT GGTGTTCCAC ACGGGTGCCG GCATCAGCAC TGCCTCTGGC
 241 ATCCCCGACT TCAGG[illegible]GGTCC CCACGGAGTC TGGACCATGG AGGAGCGAGG TCTGGCCCCC
 301 AAGTTCGACA CCACCTTTGA GAGCGCGCGG CCCACGCAGA CCCACATGGC GCTGGTGCAG
 361 CTGGAGCGCG TGGGCCTCCT CCGCTTCCTG GTCAGCCAGA ACGTGGACGG GCTCCATGTG
 421 CGCTCAGG||CT TCCCCAGG[illegible]GA CAAACTGGCA GAGCTCCACG GGAACATGTT TGTGGAAGAA
 481 TGTGCCAAGT GTAAGAC[illegible]GCA GTACGTCCGA GACACAGTCG TGGGCACCAT GGGCCTGAAG
 541 GCCACGGGCC GGCTCTGCAC CGTGGCTAAG GCAAGGGGGC TGCGAGCC||TG CAGGGGAG[illegible]AG
 601 CTGAGGGACA CCATCCTAGA CTGGGAGGAC TCCCTGCCCG ACCGGGACCT GGCACTCGCC
 661 GATGAGGCCA G[illegible]CAGG||AACGC CGACCTGTCC ATCACGCTGG GTACATCGCT GCAGATCCGG
 721 CCCAGCGGGA ACCTGCCGCT GGCTACCAAG CGCCGGGGAG GCCGCCTGGT CATCGTCAAC
 781 CTGCAGCCCA CCAA||GCACG[illegible]A CCGCCATGCT GACCTCCGCA TCCATGGCTA CGTTGACGAG
 841 GTCATGACCC GGCTCATGGA GCACCTGGGG CTGGAGATCC CCGCCTGGGA CGGCCCCCGT
 901 GTGCTGGAGA GGGCGCTGCC ACCCCTGCCC CGCCCGCCCA CCCCCAAGCT GGAGCCCAAG
 961 GAGGAATCTC CCACCCGGAT CAACGGCTCT ATCCCCGCCG GCCCCAAGCA GGAGCCCTGC
1021 GCCCAGCACA ACGGCTCAGA GCCCGCCAGC CCCAAACGGG AGCGGCCCAC CAGCCCTGCC
1081 CCCCACAGAC CCCCCAAAAG GGTGAAGGCC AAGGCGGTCC CCAGCTGACC AGGGTGCTTG
1141 GGGAGGGTGG GGCTTTTTGT AGAAACTGTG GATTCTTTTT CTCTCGTGGT CTCACTTTGT
1201 TACTTGTTTC TGTCCCCGGG AGCCTCAGGG CTCTGAGAGC TGTGCTCCAG GCCAGGGGTT
1261 ACACCTGCCC TCCGTGGTCC CTCCCTGGGC TCCAGGGGCC TCTGGTGCGG TTCCGGGAAG
1321 AAGCCACACC CCAGAGGTGA CAGCTGAGCC CCTGCCACAC CCCAGCCTCT GACTTGCTGT
1381 GTTGTCCAGA GGTGAGGCTG GGCCCTCCCT GGTCTCCAGC TTAAACAGGA GTGAACTCCC
1441 TCTGTCCCCA GGGCCTCCCT TCTGGGCCCC CTACAGCCCA CCCTACCCCT CCTCCATGGG
1501 CCCTGCAGGA GGGGAGACCC ACCTTGAAGT GGGGGATCAG TAGAGGCTTG CACTGCCTTT
1561 GGGGCTGGAG GGAGACGTGG GTCCACCAGG CTTCTGGAAA AGTCCTCAAT GCAATAAAAA
1621 CAATTTCTTT CTTGCAAA
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Homo sapiens SIRT7

ACCESSION NM_016538
VERSION NM_016538.1 GI:7706711

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   1 GCGGAAGCGG AAGAGCAGGT CTCCAGGGGA GCGATGGCAG CCGGGGGTCT GAGCCGCTCC
  61 GAGCGCAAAG CGGCGGAGCG GGTCCGGAGG TTGCGGGAGG AGCAGCAGAG GGAGCGCCTC
 121 CGCCAG[N]GTGT CGCGCATCCT GAGGAAGGCG GCGGCGGAGC GCAGCGCCGA GGAGGGCCGG
 181 CTGCTGGCCG AGAGCGCGGA CCTGGTAACG GAGCTGCAGG GCCGGAGCCG GCGGCGCGAG
 241 GGCCTGAAGC GGCGGCAGGA GGAG[N]GTGTGC GACGACCCGG AGGAGCTGCG GGGGAAGGTC
 301 CGGGAGCTGG CCAGCGCCGT CCGGAACGCC AAATACTTGG TCGTCTACAC AGGCGCGGGA
 361 ATCAGCACG[N]G CAGCGTCTAT CCCAGACTAC CGGGGCCCTA ATGGAGTGTG GACACTGCTT
 421 CAGAAAGGGA GAAGCGTTAG[N] TGCTGCCGAC CTGAGCGAGG CCGAGCCAAC CCTCACCCAC
 481 ATGAGCATCA CCCGTCTGCA TGAGCAGAAG CTG[N]GTGCAGC ATGTGGTGTC TCAGAACTGT
 541 GACGGGCTCC ACCTGAGGAG TGGGCTGCCG CGCACGGCCA TCTCCGAGCT CCACGGGAAC
 601 ATGTACATTG AA[N]GTCTGTAC CTCCTGCGTT CCCAACAGGG AGTACGTGCG GGTGTTCGAT
 661 GTGACGGAGC GCACTGCCCT CCACAGACAC CAGACAGGCC GGACCTGCCA CAAGTGTGGG
 721 ACCCAGCTGC GGGACACCAT TGTGCACTTT GGGGAGAGGG GGACGTTGGG GCAGCCTCTG
 781 AACTGGGAAG CGGCGACCGA GGCTGCCAGC AGAGCAGACA CCATCCTGTG TCTAGGGTCC
 841 AGCCTGAAG[N]G TTCTAAAGAA GTACCCACGC CTCTGGTGCA TGACCAAGCC CCCTAGCCGG
 901 CGGCCGAAGC TTTACATCGT GAACCTGCAG[N] TGGACCCCGA AGGATGACTG GGCTGCCCTG
 961 AAGCTACATG GGAAGTGTGA TGACGTCATG CGGCTCCTCA TGGCCGAGCT GGGCTTGGAG
1021 ATCCCCGCCT ATAGCAG[N]GTG GCAGGATCCC ATTTTCTCAC TGGCGACTCC CCTGCGTGCT
1081 GGTGAAGAAG GCAGCCACAG TCGGAAGTCG CTGTGCAGAA GCAGAGAGGA GGCCCCGCCT
1141 GGGGACCGGG GTGCACCGCT TAGCTCGGCC CCCATCCTAG GGGGCTGGTT TGGCAGGGGC
1201 TGCACAAAAC GCACAAAAAG GAAGAAAGTG ACGTAATCAC GTGCTCGATG AAGAACAGTT
1261 GGCACTTTGC AGATGGCCAG TGTCACGGTG AAGGCTGGGT TGCCCCCACG GGTCTAGGGA
1321 GAACGAACTC TTTGGGGATG ACATTTTCAC CGTGACATTT TTAGCCATTT GTCCTTGAGG
1381 AAGCCCCTTG CACTGCTGCG GTTGTACCCT GATACGGCCT GGCCATCGAG GACACCTGCC
1441 CATCCGGCCT CTGTGTCAAG AGGTGGCAGC CGCACCTTTC TGTGAGAACG GAACTCGGGT
1501 TATTTCAGCC CCGGCCTGCA GAGTGGAAGC GCCCAGCGGC CTTTCCTCGC TCACCAGGCC
1561 AGTCTCAGGG CCTCACCGTA TTTCTACTAC TACTTAATGA AAAAGTGTGA ACTTTATAGA
1621 ATCCTCTCTG TACTGGATGT GCGGCAGAGG GGTGGCTCCG AGCCTCGGCT CTATGCAGAC
1681 CTTTTTATTT CTATTAAACG TTTCTGCACT GGCAAAAA
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Appendix 2

Determination of Protein Concentration in Prepared Samples

Before performing immunoblotting, it was necessary to determine the concentration of protein present in the samples to be tested. Knowing this enabled experimental consistency, as the same volume of protein was used from all the samples. The method used was Bio-Rad's protein assay, which was based on the Bradford dye-binding procedure (Bradford 1976), and involves a colorimetric assay for measuring total protein concentration.

Samples were prepared for a low-concentration assay in disposable cuvettes (Gibco). First, 200µl of Bio-Rad Protein Assay Reagent (Bio-Rad) was added to the cuvette, followed by 795µl of dH2O. Subsequently 5µl of protein sample was included to the mix. The solution was thoroughly mixed with a pipette to ensure even distribution of the protein for an accurate concentration reading. In order to have something to compare the concentration of the samples with, protein standards were prepared using Bovine Serum Albumin (BSA) (Sigma). The BSA was supplied at a concentration of 2mg/ml, but for the purpose of this study it was diluted to 1mg/ml with dH2O. One reference and seven protein standards were prepared in cuvettes as follows.

Volume of 1mg/ml BSA required (ml)	**Volume of dH_2O required (ml)**
0 (REFERENCE)	800
1	799
5	795
10	790
15	785
20	780
25	775
50	750

To each of these was added 200ml of Bio-Rad Protein Assay Reagent. A gradual increase in the intensity of the blue colour was observed in parallel to the increasing concentration of the standards. Once prepared the reference and standards were used to calibrate the spectrophotometer (Bio-Rad) Protein 595 assay programme. This assay is based on a colour change of Coomassie brilliant blue G-250 dye in response to the binding of the dye

to basic and aromatic acid residues. Protein concentration was determined due to the change in the absorbance of the coomassie dye when bound to protein. When bound it has an absorption spectrum maximum of 595nm, whereas unbound it has an absorbance maximum of 470nm. Therefore, the increase of absorbance at 595nm is proportional to the amount of protein within the sample.

The optical density at 595nm (O.D. 595nm) was measured for the reference and the seven protein standards. The O.D 595 was then read for all the samples and the concentration of protein present generated from the standards concentration. The spectrophotometer calculated the amount of protein (μg/ml) present, but the theory behind it involves plotting a graph of absorbance at 595nm against protein concentration of standards. This standard curve is then used to determine the concentration of the protein present in the samples from its O.D. 595 value.

However, the concentration (μg/ml) was calculated from a diluted protein sample (1:200). From this the final concentration in mg/ml was determined as follows:

Protein reading (μg/ml) x 0.2 = Final protein concentration (mg/ml)

For western blotting either 25μg or 40μg of protein was used and the volume of sample required was calculated from the final concentration.

Appendix 3

Validation experiment for SIRT1, SIRT2, SIRT3, SIRT7 in relation to 18S (From Nadeem Ashraf experiment).