University of the West of England

An investigation in to the role of fatty acid binding protein-7, insulin like growth factor binding protein-2 and phosphatase and tensin homolog in triple negative breast cancer; *in vitro* and *in vivo*.

Sarah Joan Rose Dean

Faculty of Health and Life Sciences

2014

A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol, for the degree of Doctor of Philosophy.

In collaboration with:

Acknowledgements

First of all I would like to thank my supervisors Prof. Anthony Rhodes and Dr Claire Perks for not only their technical support and ideas but their moral support, helping me to undertake this work.

Thank you to the University of the West of England and HEFCE for funding this work, allowing it to take place.

Thank you to the University of Bristol and The University of Malaya for their collaboration especially Prof Cheng Har Yip and her colleagues supplying the tissue samples for this work.

A big thank you to Dr Caroline Jarrett for helping to get the western blot for FABP7 “cracked” and to everyone else at the University of Bristol for making me feel welcome and allowing me use of the facilities.

Everyone in 2G26 (past and present) has been a great support both emotionally and with my research; I have made some wonderful friends for life thank you all- you know who you are!

Another thank you to everyone in the Centre for Research at Biosciences and the Faculty of Health and Life sciences who has helped me especially Dr Rachel Hagen who has stepped up to help and advice on both laboratory and written work.

To my friends and family including my sister Helen, and brother-in-law Steve, thank you for your love and support over the last 3 and a half years.

To my Mum and Dad, I would not have been able to do this without you. Thank you. Your unfaltering love and support has helped get me through the hardest times and I will forever be grateful; lots of love to you both. I want to continue to make you proud- it looks like I will be wearing one of those robes!

My dear “fuzz butts” all my animals past and present, you have been a welcome distraction.

Finally, Lee- you have been through this with me and seen me every day and celebrated in the ups and picked me up in the downs. Thank you! Here’s to the next 4 years and beyond, I hope they are full of happy times, reaping the rewards and perhaps seeing some more of the world!

Abstract

Introduction

Triple negative breast cancers are defined by their lack of expression of HER, oestrogen receptors and progesterone receptors. They account for around 10-24% of cases. To define a cancer by the biomarkers it does not express is unsatisfactory. Triple negative breast cancer has been linked to aspects of metabolism and metabolic disorders such as diabetes. There are several biomarkers that are of interest and overlap in both their roles in breast cancer and in aspects of metabolism. Fatty acid binding protein 7 (FABP7) is one of 9 FABPs that is involved in the transport, solubilisation and regulation of metabolism of various fatty acids. Expression profiling and immunohistochemistry (IHC) studies have identified FABP7 to be over-expressed in a subtype of breast cancer that can be considered almost synonymous with triple negative breast cancer; basal-like breast cancer, so called because it expresses cytokeratins that are characteristic of basal epithelial cells. The role of FABP7 in breast cancer is not fully understood and studies have given conflicting results in regards to the relation to prognosis. Evidence suggests that FABP7 can be regulated by methylation acetylation and exposure to fatty acids. Insulin like growth factor binding protein-2 (IGFBP-2) is a member of the IGF-axis that is responsible for altering cell growth and metabolism. IGFBP-2 has been found to be over-expressed in many cancers including those of the prostate and breast. Phosphotensin homolog (PTEN) is a tumour suppressor gene that is responsible for dephosphorylating PIP_3 to inhibit the Akt pathway and thus inhibit cell growth and promote apoptosis. IGFBP-2 has IGF independent actions; it can down-regulate PTEN through binding of an integrin receptor and therefore have mitogenic and anti-apoptotic effects.

Aims

To study the expression of the metabolic biomarkers FABP7, IGFBP-2 and PTEN in clinical cases of Malaysian TN breast cancer. To use appropriate cell lines in order to more fully understand whether epigenetic mechanisms and FAs regulate FABP7 expression. To over-express FABP7 in a breast cancer cell lines and to further understand the role of FABP7 in breast cancer.

Methods

IHC was used to assess FABP7, PTEN and IGFBP-2 expression in a cohort of triple negative breast cancer cases. FAs, a demethylation agent-AZA and a histone deacetylase inhibitor-TSA were used

to investigate what regulated FABP7 in cell lines. Over-expression experiments were used to understand the effect of FABP7 in breast cancer cell lines.

Results

FABP7 expression in patient samples was associated with lower grade, basal-like phenotype, FAS expression and although not significant, improved patient survival. Treatment of BT-20 and MDA-MB-231 cell lines with AZA and TSA resulted in increases in FABP7 mRNA expression. Fatty acid treatment led to changes in FABP7 mRNA expression. Combinations of AZA and fatty acids gave large increases in FABP7 mRNA expression. Over-expression of FABP7 in BT-20 cells resulted in increased cell viability and although not significant changes in expression of survivin, caspase 9 and their splice variants. IGFBP-2 expression was associated with poor patient survival though this was not significant. PTEN loss was a frequent event in the cohort of triple negative breast cancer cases; 48.3% of cases had PTEN loss. PTEN loss was associated with poor patient survival though this was not significant. PTEN loss was associated with expression of IGFBP-2.

Discussion & Conclusions

FABP7 is likely to play a role in patient survival as demonstrated in the patient samples. FABP7 over-expressing BT-20 cells tended to have increased survivin FL and ΔEX3 expression. Both increased mitochondrial activity and survivin expression have been found to be associated with improved prognosis in breast cancer and this may explain some mechanisms by which FABP7 results in better prognosis in the TN breast cancer cases in this study. Since FABP7 mRNA expression was not increased to fold changes comparable to oestrogen receptor re-expression after AZA and TSA treatment, it is unlikely that the FABP7 gene is methylated or regulated by acetylation. It is possible that there are genes upstream of FABP7 such as transcription factors that are regulated my methylation and acetylation and therefore impact on FABP7 expression after treatment with AZA and TSA. This is the first study that demonstrates the significant relationship between IGFBP-2 expression and PTEN loss in patient samples. PTEN loss is a frequent event in TN breast cancer. IGFBP-2 and PTEN loss may be useful markers of prognosis in TN breast cancer.

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Abbreviations

5-aza-dC	*5-Aza-2'*-deoxycytidine
9cis-RA	9-cis-retinoic acid
AA	Arachidonic Acid
ACC-α	Acetyl CoA Carboxylase
AMPK	AMP activated protein Kinase
AZA	*5-Aza-2'*-deoxycytidine
BLBC	Basal-like breast cancer
BLBP/BFABP/FABP-B (FABP7)	Brain-Lipid Binding Protein
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CDB	Cell Dissociation Buffer
cDNA	Complementary DNA
chIP	Chromatin-immuno precipitation
CI	Confidence interval
CISH	Chromogenic *in situ* hybridisation
CK	cytokeratin
COX2	Cyclooxygenase 2
Ct	Cycle Threshold
DAB	Diaminobenzidine tetrahydrochloride
DCIS	Ductal Carcinoma *in situ*
DHA	Docosahexaenoic Acid

dH_2O	Distilled water
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyltransferase
DSBs	Double strand breaks
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Eagle’s Minimum Essential Medium
ER	Oestrogen Receptor
ERE	ER response element
FA	Fatty Acid
FAs	Fatty Acids
FABP	Fatty Acid Binding Protein
FABPs	Fatty Acid Binding-Proteins
FAS	Fatty Acid Synthase
FBS	Foetal Bovine Serum
FISH	Fluorescent *in situ* hybridisation
g	grams
gDNA	genomic DNA
GLUT4	Glucose transporter type 4
HAT	Histone acetyl transferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase

Her	Human Epidermal Growth Factor Receptor
HNF4	Hepatic nuclear factor 4
IAP	Inhibitor of apoptosis protein
IDC	Invasive ductal carcinoma
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IGFBPs	Insulin-Like Growth Factor Binding-Proteins
IHC	Immunohistochemistry
LB	Luria-Bertani
LG	L-Glutamine
LOH	Loss of heterozygosity
MeCP	Methyl-CpG binding proteins
mL	millilitre
µL	microlitre
MDGI (FABP3)	Mammary Derived Growth Inhibitor
MET	Mesenchymal-epithelial transition
MRG (FABP7)	Mammary Derived Growth Inhibitor Related Gene
mRNA	messenger RNA
NICE	National Institute for health and Care Excellence
NF-I	Nuclear factor-I
ng	nanogram
NT	no-template
NPI	Nottingham prognostic index

OR	Odds ratio
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxykinase
Pen-Strep	Penicillin-Streptomycin
PIP_2	Phosphatidylinositol (4,5)-trisphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptors
PPRE	PPAR response element
PR	Progesterone Receptor
PTEN	Phosopho-Tensin homolog
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative PCR
RIP	Receptor interacting protein
ROS	Reactive oxygen species
RSR	Relative survival rate
-RT	minus reverse transcriptase
RT	Reverse Transcription
RXR	Retinoic X receptor
SDS	sodium dodecyl sulphate
SEM	Standard error of the mean

siRNA	Small interfering RNA
SISH	Silver enhanced *in situ* hybridisation
SFM	Serum Free Media
SREBP	Sterol regulatory element-binding protein
SSBs	Single strand breaks
STAT	Signal Transducer and Activator of Transcription
STRP	Staurosporine
TAE	Tris-acetate-EDTA
TBS	Tris-buffered Saline
TEMED	tetramethylethylenediamine
TN	Triple Negative
TSA	Trichostatin A
Tris	Tris hydroxymethylamine
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 The Breast and Breast Cancer

Breast cancer is the most common type of cancer in the UK with over 45,000 new cases each year (Cancer Research UK, 2010). Worldwide it is one of the leading causes of cancer deaths with approximately 519, 000 dying each year from the disease with incidence increasing with age (World Health Organisation, 2009).

The human breast is comprised of fatty and fibrous tissues or stroma, with ducts and lobes. The lobes, also called glands produce milk during lactation (figure 1.1). The breasts, or mammary glands, are one of the few tissues that are subjected to major morphological changes during developmental phases such as puberty and pregnancy. For instance, nulliparous women had significantly less lobular development and differentiation than women who had children (Russo *et al.*, 2005). The network of ducts are formed before birth by branching out throughout the fat pad; they comprise of a basal layer of myoepithelial cells and a layer of specialized epithelial cells (Dontu *et al.*, 2003) (figure 1.1). Epithelial, mesenchymal and stromal interactions occur under the influence of hormones from the pituitary and the ovaries, such as oestrogen and progesterone; these interactions and hormones are essential for breast development during life (Robinson *et al.*, 1999; Howard & Gusterson, 2000).

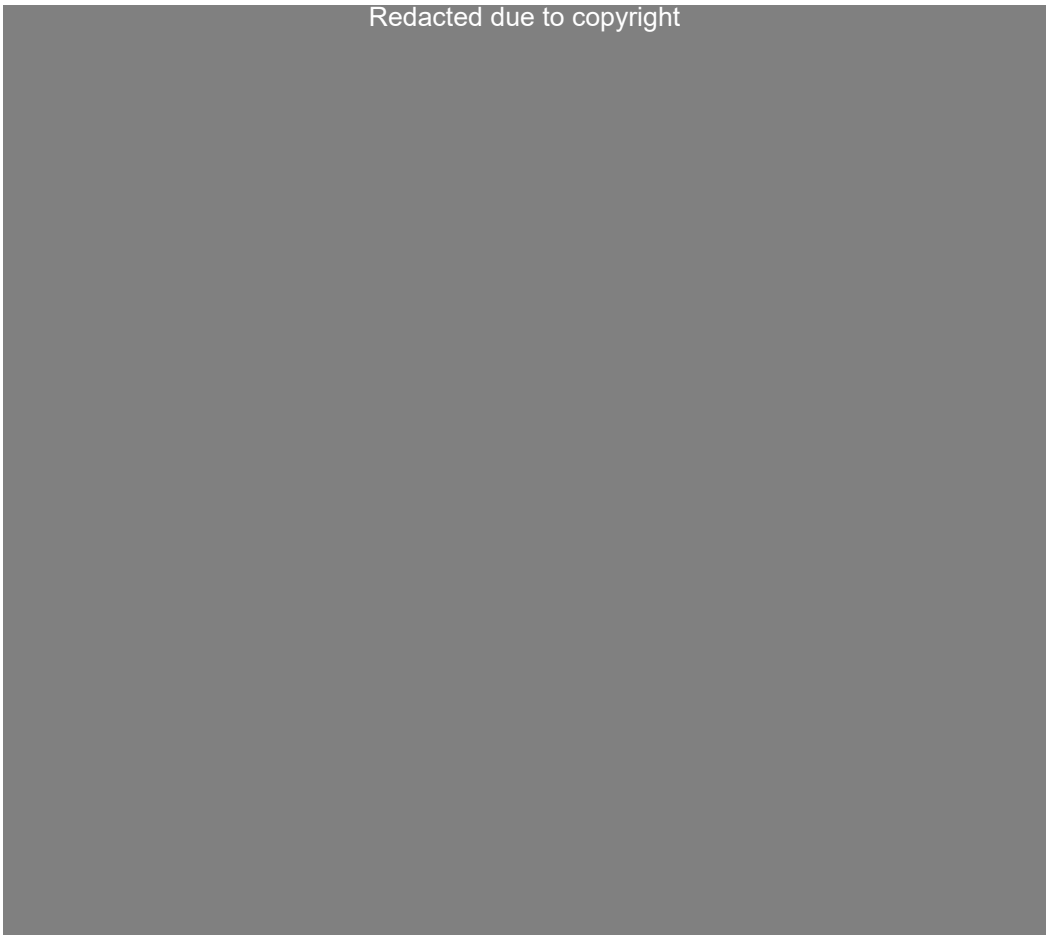


Figure 1.1 A diagram showing the main structures of the breast and *in situ* and invasive breast cancer

The breast is comprised of fatty tissue and milk ducts. The lobes or glands produce milk during lactation. Ducts comprise of a basement membrane with a layer of myoepithelial or basal cells and a layer of epithelial cells that line the inside of the ducts, a cross sectional representation is shown above. Breast cancer can be contained within the duct, *in situ*, or invasive. Diagram adapted from http://cancerhelp.cancerresearchuk.org

Breast cancer is a heterogeneous group of diseases that usually originate from the epithelial cells lining the ducts or glands, however, it can also develop from the layer of myoepithelial cells in the duct or within the surrounding tissues of the glands and ducts. Breast cancer originating from the ducts can be *in situ* (DCIS) i.e. contained within the duct or invasive where tumour cells have invaded outside of the duct (figure 1.1). The multistep theory of breast cancer development helps explain how breast cancer arises and progresses (Thompson *et al.*, 1992; Beckmann *et al.*, 1997);

a schematic representation is shown in figure 1.2. More recently mechanisms other than an accumulation of genetic abnormalities have been shown to be important in carcinogenesis and tumour progression. For example epigenetic alterations such as methylation or acetylation, leading to changes in gene expression, and posttranslational modification of proteins that lead to differences in function or activity, are important factors in tumourigenesis (Cancer Genome Atlas Network, 2012; Sharma *et al*., 2010).

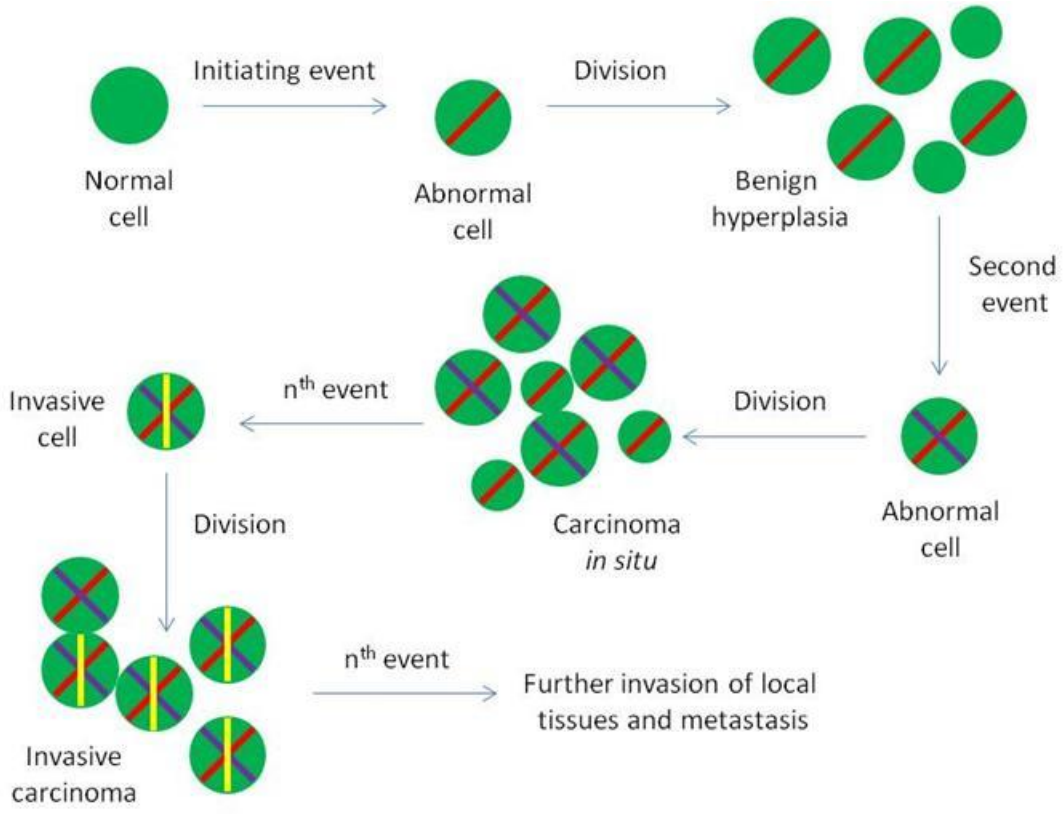


Figure 1.2 A schematic representation of the multi-step theory of carcinogenesis

This figure represents the multistep theory of carcinogenesis. It begins with an initiating event, for example, a mutation in an oncogene or tumour suppressor gene. The abnormal cell then divides at a faster rate than the normal cells leading to benign hyperplasia. Further mutations occur eventually leading to highly abnormal and invasive cells.

The initiating event in the development of breast cancer can be caused by inherited gene mutations, such as mutations in *BRCA1* or *2* (discussed in section 1.3.5); *BRCA1* and *2* mutations are frequent in familial breast cancer, i.e. breast cancer that can be traced through generations of women. It is estimated that carriers of *BRCA1* or *BCRA2* mutations have around an 80% risk of developing breast cancer by age 70. However *BRCA1* and *BRCA2* mutations are present in only around 5-10% of all breast cancers (Ford *et al.*, 1998; Oesterreich & Fuqua, 1999; Peto *et al.*, 1999). The remaining breast cancers are sporadic and can be initiated by environmental exposures such as diet and lifestyle which are to be discussed further; as mutations accumulate, the abnormal cells become more invasive and grow out of the ducts. Due to the proximity of the breast to the lymphatic system, breast cancer can invade other tissues in the body through this network.

1.2 Diagnosis and screening of breast cancer

In the UK there is a breast cancer screening programme. Women between the age of 50 and 70 are invited for a mammography every 3 years, by the year 2016 this will be extended to ages 47 to 73 (Cancer Research UK). A mammography is an X-ray of the breast that can help detect calcification or other changes in tissue density that are indicative of breast cancer. In addition to this women are educated to check their breasts for lumps or changes on a regular basis. An early study found that breast self examination was associated with a lower stage of cancer at the time of diagnosis compared to women who never undertook self examination; it was predicted that breast self examination would lead to a 10% reduction in 5 year mortality rates (Feldman *et al.*, 1981). However there is much debate over the use of mammography and reduction in mortality rates. One study found by looking over a 20 year period in Sweden before and after screening was introduced breast cancer mortality rates significantly reduced in the women who were routinely screened; this was not due to changes in incidence or clinical practice (Tabar *et al.*,

2003). Another study in Denmark had similar findings with a 25% reduction in mortality due to screening (Olsen, *et al*., 2005). In contrast to this, a different group in Denmark found no reduction in mortality due to screening; they also compared their results to other countries including the UK and stated there were similar findings (Jorgensen *et al.*, 2010). Over diagnosis of breast cancer due to mammography screening is also an issue; breast cancers that would otherwise not have been detected nor given any harm to patients, as they would have died from other causes first, are treated unnecessarily (Jorgensen *et al.*, 2009; Zackrisson, *et al*., 2006). On detection of an abnormality a biopsy or fine needle aspiration is taken; these methods collect cells for microscopical examination. The samples are studied by a pathologist who can distinguish abnormal cancer cells from normal breast cells. If breast cancer is confirmed, surgery is undertaken to remove the tumour. The surgery can be a lumpectomy, where just the tumour and some surrounding breast tissue is removed, or a mastectomy, where the whole breast is removed. The expression of markers such as oestrogen receptors, progesterone receptors and human epidermal growth factor receptor are used to determine targeted therapy options and prognosis; these are to be discussed further.

1.3 Breast cancer types and incidence

The most common type of breast cancer is ductal carcinoma arising from the epithelial cells or less commonly the myoepithelial cells of the duct. Approximately 70-80% of breast cancers diagnosed in the UK are invasive ductal carcinoma (Cancer Research UK, 2010). Breast cancer can also arise in the lobes from epithelial cells, as in figure 1.1; like ductal carcinoma, lobular carcinoma can be *in situ* or invasive. Rarer types of breast cancer include medullary, so called because the macroscopic appearance resembles the medulla in the brain; mucinous, where cancer cells are surrounded by mucin, and tubular breast cancer, named after the tube-like structures observed under the microscope. As mentioned breast cancer can also develop within the tissues

surrounding the breast ducts and glands; this type of breast cancer is also rare accounting for less than 1% of breast cancers, it is called angiosarcoma of the breast (Gallager, 1984). These different types of breast cancer encompass histological classification; invasive types have poorer prognosis than those *in situ*; though there are other more recent means of classifying breast cancer for example at the molecular level.

Breast cancer can be classified according to stage and grade. Grade is based on microscopic appearance of the cancer cells for example, cell morphology, nuclear pleomorphism and numbers of mitotic figures; a score of 1-3 is applied depending on the extent of how abnormal the cancer cells are for each criteria; these scores are combined leading to a grade of 1-3 (Blamey *et al.*, 2007; Bloom & Richardson 1957) (table 1.1)

Tubule Formation	**score**
>75%	1
10-75%	2
less than 10%	3
Nuclear Pleomorphism	**score**
Small uniform cells	1
moderate increase in size and variation	2
marked variation	3
Mitotic count (per 10 high power fields)	**score**
up to 7	1
7-14	2
15 or more	3
	total score
Grade 1	3-5
Grade 2	6-7
Grade 3	8-9

Table 1.1 Breast cancer grading

Breast cancer is graded according to tubule formation, nuclear pleomorphism and mitotic count; the score for each of these is combined to give the final grade. Information from Blamey *et al.*, (2007); Bloom & Richardson (1957) and Elston & Ellis, (1991).

Stage takes into account tumour size and extent of invasion; (table 1.2). Breast cancers with lower grade and stage tend to have a better outcome; for instance in a study by Henson *et al*., (1991); the outcomes of over 20,000 breast cancer cases were compared to grade and stage; grade 1 breast cancers had an overall survival rate of 93% compared to grade 3 with 65% survival at 5 years (Henson *et al.*, 1991).

Stage	Features
1	<2cm in size. No invasion anywhere.
2A	<2cm in size some lymph node involvement OR 2-5cm in size no lymph node involvement OR No tumour in breast but tumour in lymph nodes under armpit.
2B	<5cm in size and lymph node involvement OR >5cm in size but no lymph node involvement.
3A	No tumour in breast but clumps of tumour cells in lymph nodes OR <5cm in size and lymph nodes contain clumps of tumour cells OR >5cm in size lymphnodes contain clumps of cells but no further invasion.
3B	Tumour has attatched to chest wall or skin; lymph nodes may or may not contain tumour cells but no metastasis.
3C	Any size; invasion in lymph nodes and under breast bone or to nodes around the collar bone. No further invasion.
4	Any size and lymph node involvment; tumour has invaded other tissues of the body, for example lungs, liver or bone.

Table 1.2 Staging criteria for breast cancer tumours
Breast cancer is staged according to the tumour size (T), amount of invasion to lymph nodes (N) and surrounding tissues (M); the higher the stager the poorer the prognosis. This table represents the current 'TNM' staging criteria (information from Cancer Research UK)

1.3.1 Hormone receptor positive breast cancer

Breast cancer can be classified according to the positivity of the hormone receptors for oestrogen (ER) and progesterone (PR) as demonstrated by immunohistochemistry (IHC). The proportion of hormone receptor over-expressing cases is between 70-80% but varies with age, with the highest proportion in western populations being in those over 65; individuals may also not necessarily be positive for both ER and PR as demonstrated using IHC (Rhodes *et al.*, 2000; Li *et al.*, 2003; Rakha *et al.*, 2007b; Rhodes & Jasani, 2009).

Oestrogen has been implicated in carcinogenesis in a number of circumstances (figure 1.3); oestrogen metabolites can induce DNA damage though binding to guanine or adenine to form unstable DNA adducts, or by oxidative damage to DNA (Yager & Davidson, 2006). Oestrogen can also drive carcinogenesis in the breast through activation of the ER and downstream signalling pathways; the activated ER can also bind to transcription factors to activate them and to ER response elements in DNA to recruit or prevent binding of activators or co—activators; as a result gene expression is altered. For example, ER can interact with transcription factors including activating protein 1 (AP1), specificity protein 1 (SP1) and nuclear factor-κB (NF—κB) (Fox *et al.*, 2009; Thomas *et al.*, 2010). Breast cancer cells that express ER are over stimulated by oestrogen and so apoptosis is inhibited and proliferation is promoted. ER over-expression can occur through hypomethylation of the gene or, in a small number of cases, amplification of the gene (Fantl *et al.*, 1990; Piva *et al.*, 1990).

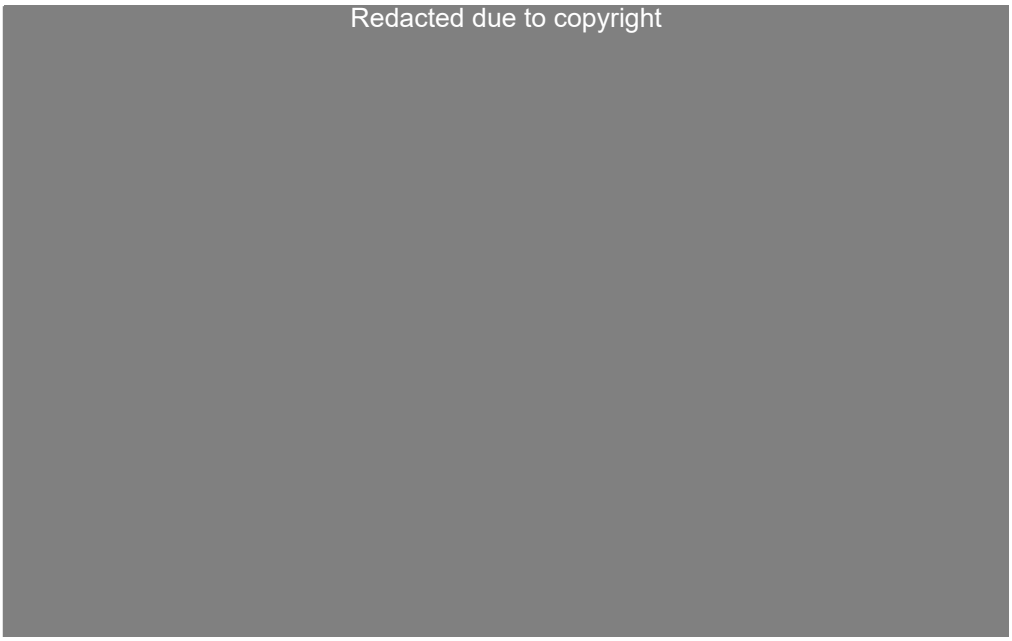


Figure 1.3 The mechanisms of oestrogen carcinogenesis

Oestrogen can drive breast cancer through either its metabolites damaging DNA and altering expression through direct binding, or through binding to the ER to directly or indirectly activate or inhibit transcription of genomic or mitochondrial DNA. This leads to increased proliferation and decreased apoptosis. Diagram adapted from (Yager & Davidson, 2006).

Patients with ER positive breast cancer tend to have better survival than patients with ER negative breast cancer. One early study found that 2 year survival rates were around 70% in ER positive patients compared to approximately 60% survival in ER negative patients (Bishop *et al.*, 1979); another study found that beyond 2 years survival differences between ER positive and negative patients was more marked (Stewart *et al.*, 1981). However in more recent years, overall breast cancer survival rates have improved; in 2009 the five year survival was 85.1% (Cancer Research UK). This improvement is a result of improved therapy which will be discussed further. ER positive tumours tend to metastasize to the bone unlike ER negative tumours which spread more often to the brain and liver (Stewart *et al.*, 1981). More recently metastases in ER positive breast cancers have been linked to acquired regions of ER binding and that FOXA1, a DNA binding protein, reprograms the binding of ER in metastatic breast cancer (Ross-Innes *et al.*, 2012). Initially, PR status was used as an indicator of ER function as the receptors are expressed in response to ER signalling pathway activation (Lange, 2008). Breast cancers can be PR positive and ER negative; it was originally thought this may be a technical artefact but recent work has demonstrated otherwise (Ng *et al*., 2012). More recently it has been found that the PR is involved in carcinogenesis independently of the ER signalling pathways; PR signalling can sensitize cells to growth factors. In addition post-translational modifications, such as phosphorylation of PRs, are important in cell signalling within breast cancer (Knutson *et al.*, 2012). PR is also useful for predicting mortality in breast cancer; a retrospective study found that PR positivity was associated with an improved prognosis and was particularly useful in predicting outcome when combined with factors such as ER and lymph node status in patients that had received endocrine therapy (Bardou *et al.*, 2003).

1.3.2 Treatment of hormone receptor positive breast cancer

The main drug utilised that targets hormone receptor positive breast cancer is tamoxifen. Tamoxifen can be used in conjunction with chemotherapeutic agents and works by binding to the ER (figure 1.4), therefore preventing activation of proliferative and anti-apoptotic genes. A recent clinical trial evaluated the effectiveness of tamoxifen combined with everolimus, a drug that inhibits mTOR signalling pathways. The combination of tamoxifen with everolimus lead to improved overall survival and increased time to breast cancer progression than tamoxifen used alone (Bachelot *et al*., 2012). Due to the effectiveness of tamoxifen against breast cancer preventative use has been suggested; one study gave tamoxifen or a placebo to women for 5 years, they found that the incidence of breast cancer in the tamoxifen group was 24.8 per 1000 women compared to 42.5 for the placebo group (Fisher *et al.*, 2005). Another group of drugs used against hormone receptor positive breast cancer are aromatase inhibitors; they work by inhibiting the enzyme complex that converts hormones, such as testosterone, to oestrogens, thus reducing the production of oestrogen so there is less oestrogen to bind to ERs and stimulate the cancer cells (figure 1.4). It has been suggested that aromatase inhibitors can be used to prevent breast cancer, particularly in women with high plasma oestrogen levels (Goss & Strasser, 2001; Smith & Dowsett, 2003; Johnston & Dowsett, 2003).

Despite targeted therapies for ER positive breast cancers, some do not respond or become resistant to treatment. Mechanisms for this include loss of ER expression or a mutation that results in loss of ER function and tolerance of tamoxifen through increased metabolism of the drug (Ring & Dowsett, 2004).

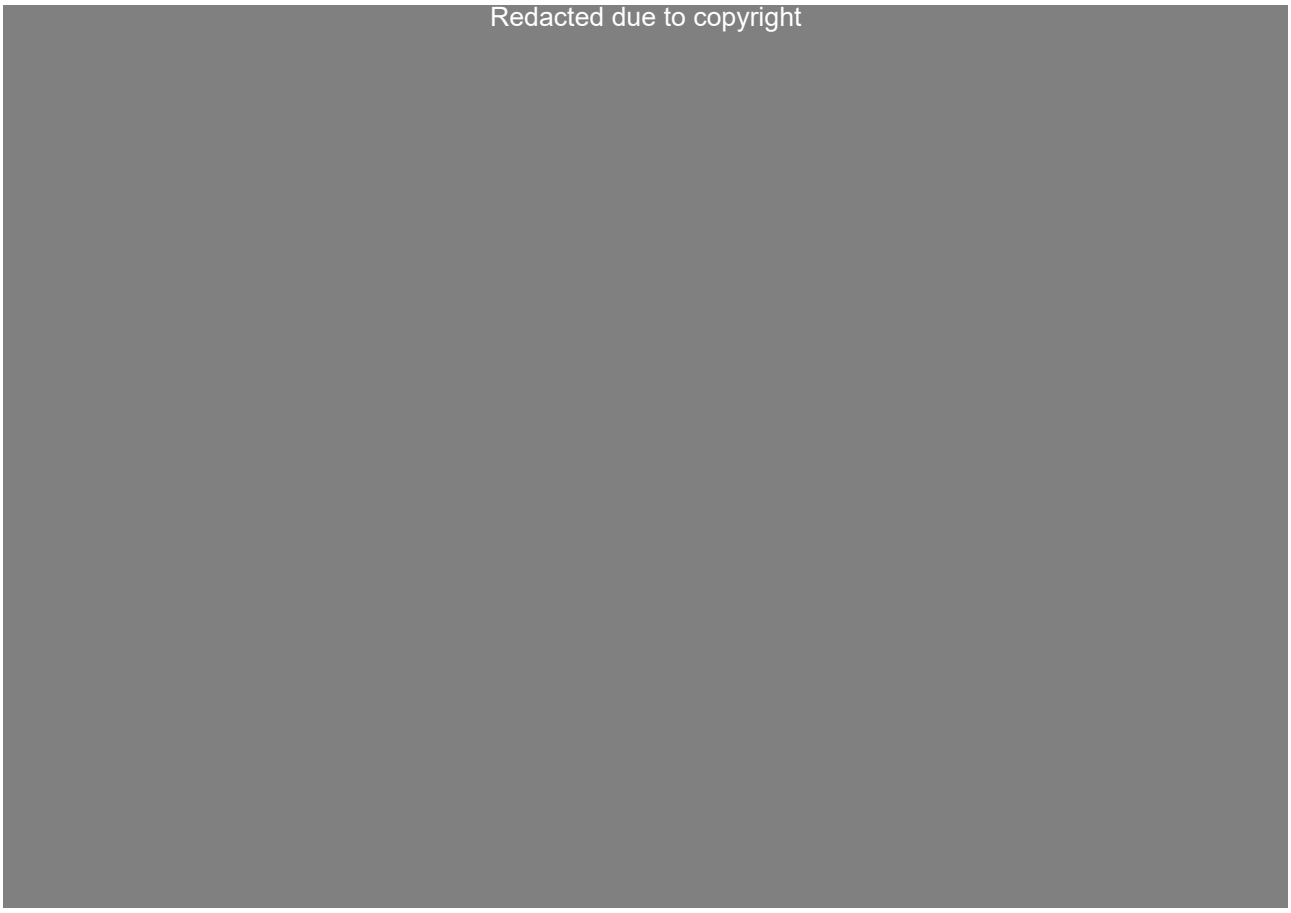


Figure 1.4 The action of targeted therapies for ER positive breast cancer

There are 2 types of targeted therapy for oestrogen receptor (ER) positive breast cancer. Tamoxifen prevents the binding of oestrogens to the ER so that in turn gene promoters that contain regions of ER binding, i.e. ER response elements (EREs), are not activated. These EREs of promoter regions are upstream of ER target genes that when activated increase cell proliferation. Aromatase inhibitors work by preventing the production of oestrogens by inhibiting the aromatase enzyme complex from converting androstenedione and testosterone to oestrogens such as oestrone and oestradiol. Both tamoxifen and aromatase inhibitors prevent activation of EREs in promoter regions upstream of ER target genes so that proliferation is inhibited. Adapted from Johnston & Dowsett (2003).

1.3.3 Human epidermal growth factor receptor 2 positive breast cancer

Human epidermal growth factor 2 (Her2) is an important biomarker used in the prognosis of breast cancer. It is an epithelial growth factor receptor (Egfr) related tyrosine kinase and is part of the HER/ErbB2/Neu family. In the normal breast, Her2 is involved with development and growth. Activation occurs through dimerisation of two Her2 receptors to form a homodimer, or through dimerisation of Her2 and another receptor from the Her family to form a heterodimer. The homodimers or heterodimers phosphorylate substances such as themselves or other signalling molecules such as Phosphoinositide 3-kinase (PI3K) (Yarden & Sliwkowski, 2001), that lead to intracellular signalling causing increased cell proliferation and survival (Yarden, 2001; Dean-Colomb & Esteva, 2008). The glycoprotein receptor is over expressed in 20-30% of invasive breast carcinomas (Slamon *et al.*, 1987; Yarden, 2001; Nunes *et al.*, 2008). *HER2/neu* is the gene that encodes Her2; it is found on chromosome 17 and is a proto-oncogene. Amplification of this gene is responsible for Her2 over-expression in 90% of Her2 positive cases (Slamon *et al.*, 1987; Popescu *et al.*, 1989). The remaining Her2 over-expressing cases are due to gene deregulation at a post-transcriptional level (Miller *et al.*, 1994). Most Her2 positive tumours are ER and PR negative and are associated with a poorer prognosis than breast cancers with hormone receptor over-expression (Tandon *et al.*, 1989; Cheang *et al.*, 2009). More recently it has been found that polymorphisms in the *IGF-I* gene can differentiate between good or poor survival within Her2 positive breast cancer (Muendlein *et al.*, 2013).

1.3.4 Treatment of Her2 positive breast cancer

Like ER and PR, Her2 over-expression can be demonstrated by IHC. Cases are scored on the amount and intensity of membrane staining; this is used to help determine response to treatment with the drug trastuzumab (herceptin). Cases that are 1+ or negative are considered not to benefit from targeted therapy whereas those that are 3+ are offered the drug (Nahta & Esteva, 2006). Equivocal cases, i.e. those with a 2+ score (figure 1.5), where membrane staining is not complete or as intense as a 3+ case, are tested further with fluorescence *in situ* hybridisation (FISH), chromogenic *in situ* hybridisation (CISH) or more recently silver enhanced *in situ* hybridisation (SISH). The FISH, CISH and SISH techniques use nucleic acid probes that bind to specific sequences of DNA or RNA is tissue sections. The probes are labelled so that they can be visualised when bound to the DNA or RNA. Using these techniques it is possible to visualise how many copies of the *HER2/neu* gene are present within the breast cancer (Hicks & Tubbs, 2005). This determines whether the *HER2/neu* gene is amplified (Dietel *et al.*, 2007; Nunes *et al.*, 2008; Shousha *et al.*, 2009). If the gene is amplified in Her2 positive equivocal cases the patient will receive trastuzumab therapy.

Figure 1.5 The range of Her2 expression in invasive breast cancer

Cases that are 0 or 1+ will not receive therapy as Her2 is not over-expressed. Cases that are 2+ have intermediate intensity staining that is present in more than 10% of the tumour but is incomplete around the cells. These cases will be subjected to *in situ* hybridisation to determine treatment. Cases that are 3+ have strong staining that is complete in greater than 10% of the tumour forming a "chicken wire" pattern. These cases are most likely to benefit from trastuzumab therapy. (Images from "latest breast cancer" website).

Trastuzumab is a monoclonal antibody that works by preventing dimerisation of the Her2 receptors with other Her2 receptors or members of the Her family such as epidermal growth factor receptor (Egfr). Trastuzumab can lead to activation of the immune system to initiate a response against the cells over-expressing Her2 and prevent the tyrosine kinase activity of Her. This prevents phosphorylation of itself and activation of other signalling molecules. These actions in turn lead to decreased proliferation and increased apoptosis (figure 1.6).

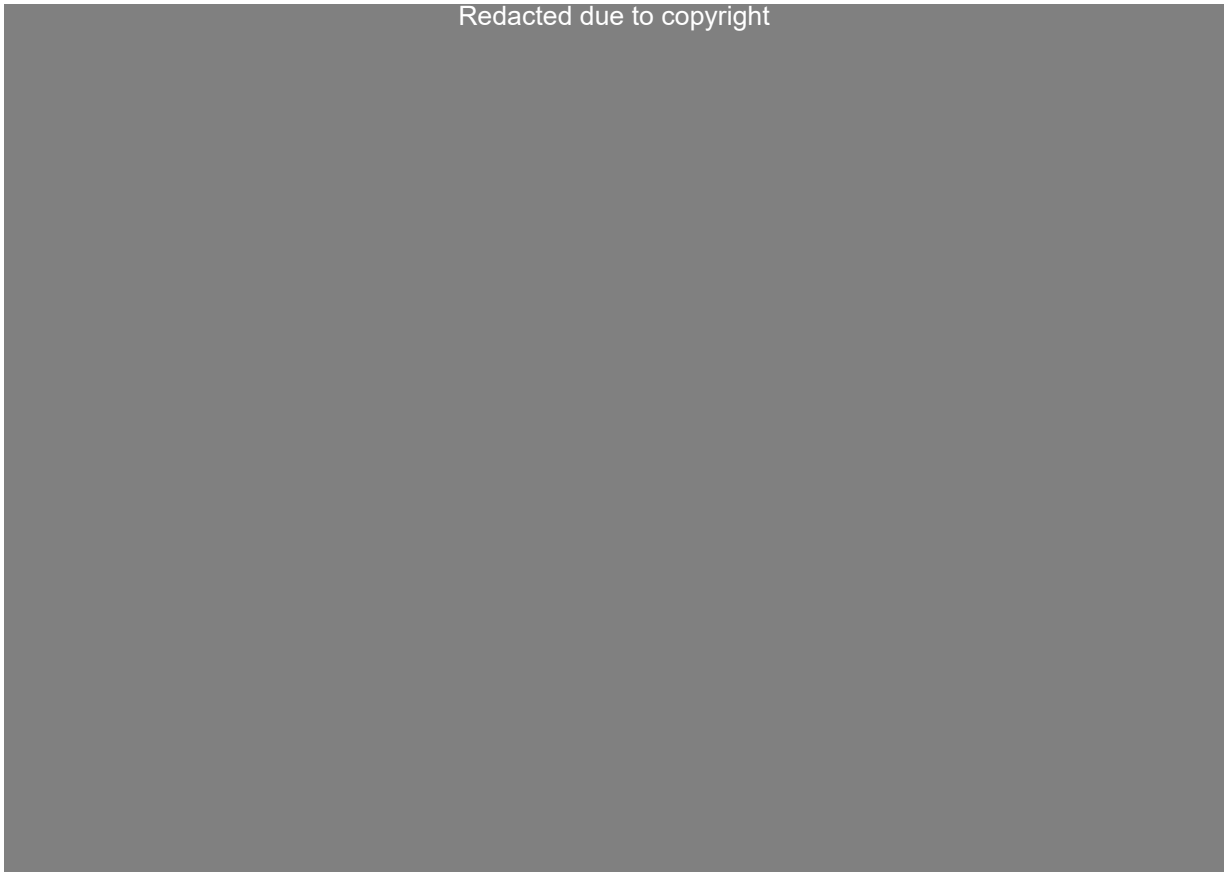


Figure 1.6 The action of trastuzumab

Trastuzumab prevents dimerisation of Her2 receptors with each other or other members of the Her2 family. This blocks activation of signalling pathways leading to induction of apoptosis and decreased proliferation. Trastuzumab can also lead to immune activation and decreased tyrosine activity to prevent phosphorylation of other Her2 and other signalling molecules. Adapted from Burstein (2005).

Trastuzumab can be used in conjunction with chemotherapeutic agents such as docetaxel, and has been shown to improve survival of breast cancer patients; this improvement was noticeable even after only one year of follow up (Stebbing *et al.*, 2000; Smith *et al.*, 2007). Recently, a clinical trial has investigated the combined use of trastuzumab and docetaxel with another monoclonal antibody that targets Her2. Pertuzumab targets a different subdomain of Her2 than trastuzumab. The findings of the trial showed that pertuzumab in combination with trastuzumab and docetaxel resulted in a reduction of breast cancer progression and increased survival compared to cases treated with only trastuzumab and docetaxel. The study highlighted the importance of blocking Her2 dimerisation with other Her family members by targeting more than one region of the Her2 receptor at once (Baselga *et al.*, 2012).

Despite over-expressing Her2, some breast cancers are resistant or become resistant to trastuzumab. One resistance mechanism is the truncation of the Her2 receptor, trastuzumab cannot bind to Her2 because the extracellular domain is not present (Pohlmann *et al.*, 2009).

Another drug used against Her2 positive breast cancer is Lapatinib, a molecule that inhibits Her2 and EGFR dimerisation thus blocking downstream signalling of these receptors. Lapatinib has been found particularly effective against breast cancers that are resistant to trastuzumab (Konecny, *et al.*, 2006).

1.3.5 Other targeted therapies for breast cancer

More recently other targeted therapies for breast cancer have been developed. One example is Avastin. Avastin is a monoclonal antibody that targets vascular endothelial growth factor (VEGF). VEGF stimulates the growth of blood vessels and can be over-expressed in many cancers including those of the breast. In a tumour, targeting VEGF is useful, as it slows blood vessel growth and thus prevents gas exchange and nutrients reaching the tumour cells (Ferrara *et al.*, 2005; Rugo,

2004). Clinical trials have shown that Avastin is effective in improving relapse free survival but not overall survival (Bear *et al.*, 2012). Due to these trial results there has been much debate over the use of Avastin; recently the National Institute and Clinical Excellence (NICE) has said it should not be used in advanced metastatic breast cancer because of the added cost as Avastin did not show any added benefit compared to current drugs alone (Cancer Research UK 2012 news).

Another group of drugs that are classed as targeted therapy are Poly (ADP-ribose) Polymerase (PARP) inhibitors. PARP is an enzyme involved in the repair of single strand breaks (SSBs) in DNA. PARP inhibitors block the repair of SSBs (Virag & Szabo, 2002) which, if left unrepaired, convert to double strand breaks (DSBs). *BRCA1* and *BRCA2* are known as breast cancer susceptibility genes because mutations in these genes result in a high probability of developing breast or ovarian cancer. *BRCA1* acts as a tumour suppressor gene and encodes a protein that is involved with maintaining genetic stability. The exact functions of *BRCA1* and the pathways it is involved with are complex; recently *Brca*1 has been implicated in the regulation of fatty acid synthesis through acetyl-coA carboxylase (ACC) (Brunet *et al.*, 2008). *Brca*1 binds to the phosphorylated or inactive form of ACC, the rate limiting enzyme in fatty acid synthesis, and stabilises phospho-ACC. If Brca1 is abnormal or present in low levels, ACC is more abundant in its active form, thus impacting on cell metabolism through fatty acid synthesis (Brunet *et al.*, 2008). The interaction of ACC with *Brca*1 occurs through protein interaction motifs, called BRCT domains; these domains can be affected when *BRCA1* is mutated (Magnard *et al.*, 2002). With regards to action on DNA repair, Brca1 and *Brca*2 interact with RAD51 to repair DNA by forming a macromolecular complex. In addition *Brca*1 is also a transcriptional regulator; reviews have outlined how *Brca*1 can bind to enzymes involved in transcription to activate them. *Brca*1 can also bind directly to promoter regions of genes; for example p21 a cell cycle regulator, to activate cell cycle checkpoints to allow DNA repair or ensure cell death if repair is not possible (Welcsh *et al.*, 2000; Yoshida & Miki, 2004). Inactivating mutations in *BRCA1* and *2* lead to an accumulation of DNA damage and cell cycle deregulation including DSB that are usually repaired by homologous recombination. PARP

inhibitors are useful in tumours that have *BRCA* inactivating mutations or where *BRCA* is lost as they prevent SSB repair so DSBs occur leading to synthetic lethality or cell death. However cells can overcome PARP inhibition when they retain one copy of *BRCA1* or become resistant through functional mutations in *BRCA* or when mutations occur in other genes that enable restoration of DNA repair (figure 1.7 and figure 1.8) (De Soto *et al*., 2006; Lord & Ashworth, 2008; Underhill *et al.*, 2011; Polyak & Garber, 2011). The pathways that PARP is involved with SSB repair and BRCA is involved in DSB are outlined in more detail in figure 1.8.

Figure 1.7 The action of PARP inhibitors in breast cancer

PARP inhibitors prevent the repair of single strand breaks (SSBs) leading to double strand breaks (DSBs) that will be lethal to tumour cells lacking Brca protein (caused by loss of heterozygosity (LOH) of the *BRCA* genes). Tumours or normal cells (that carry BRCA abnormalities) retaining one copy or a functional mutated form of *BRCA* and normal cells will be resistant to death by PARP inhibition. Figure from Polyak & Garber (2011).

Figure 1.8 The role of PARP in SSB repair and the role of BRCA in DSB repair

A: PARP is recruited to the site of the single strand break which in turn recruits the scaffold protein XRCC1. DNA polymerase β (polβ) replaces the damaged nucleotide and DNA ligase III (LigIII) is recruited to ligate the nick. If PARP is inhibited this process cannot occur and the SSB leads to DSB. B:DSBs are repaired by homologous recombination. This figure shows how BRCA is involved in the repair of DSB. Phosphorylation of BRCA1 leads to complex formation with BRCA2, BRCA1-associaated RING domain protein 1 (BARD1) and RAD51. This complex is relocated to the area of DNA replication after damage marked by proliferating cell nuclear antigen (PCNA). In normal cells this leads to DNA repair and normal cell cycle. In cells with BRCA dysfunction DNA repair is not possible; in cells with normal tumour suppressor gene expression such as p53 and cell cycle checkpoint gene expression such as p21, cell cycle arrest and apoptosis occurs. In cells with abnormal p53 or checkpoint genes cell proliferation can occur. Adapted from Arnold & Goggins (2001) and Curtin (2005).

Despite such targeted therapies some breast cancers do not respond or become resistant and currently research is being undertaken to understand why and to develop alternatives. Moreover there are a group of breast cancers that do not express ER, PR of Her2 and consequently do not respond to targeted therapy such as aromatase inhibitors, tamoxifen or trastuzumab.

1.4 Triple negative breast cancer

There are 10-24% of breast cancers that do not over-express ER, PR or Her2, so called triple-negative (TN) breast cancers due to their lack of expression of these key biomarkers. These breast cancers tend to be of a higher grade and stage than other breast cancers (Rakha *et al.*, 2007b); they also tend to have poorer survival, as in figure 1.9, and are more likely to metastasize to distant sites (Kaplan & Malmgren, 2008; Dent *et al.*, 2009; Hernandez-Aya *et al.*, 2011). Compared to other breast cancers TN breast cancers occur in a younger age group, for example a median age of diagnosis of 48 was reported in a TN cohort and another group found that TN breast cancer was more common in those under the age of 40 (Bauer *et al.*, 2007; Hernandez-Aya *et al.*, 2011).

Figure 1.9 The survival probability of triple-negative breast cancers

This graph shows the survival probability of TN breast cancers in years after diagnosis. TN patients have poorer survival probability, decreasing over time compared to other hormone receptor positive and Her2 positive breast cancer (Dent *et al.*, 2009).

TN breast cancers are less likely to be diagnosed by screening methods such mammography or ultrasound; suggestions for this include the rapid growth rate of TN tumours or differences in tumour density of TN breast cancers compared to other breast cancer types leading to difficult detection through imaging of the breast (Dent *et al.*, 2007). Currently, patients with these highly aggressive tumours have a poor prognosis, with no specific therapy available for their treatment as there is for hormone receptor and Her2 positive breast cancer (Reis-Filho & Tutt, 2008). To define TN breast cancers by markers they do not express is an unsatisfactory way of classifying and studying these important tumours. Fortunately, expression profiling has identified a group of breast carcinomas that are somewhat synonymous with triple negative breast cancer, in that the majority of them are negative for ER, PR and Her2.

1.4.1 Basal-like breast cancer

Breast cancers have been traditionally defined by their histological nature i.e. their appearance or markers they express with IHC. Molecular profiling techniques are becoming more commonplace in defining breast cancer types. Molecular profiling involves looking at protein, mRNA or DNA on a level where many comparisons are made at the same time. One technique in particular, microarray analysis, involves a microarray of DNA probes for specific genes of interest. The cDNA samples, converted from mRNA, bind to these probes and a signal is emitted reflecting the levels of expression of a gene of interest (DeRisi *et al.*, 1996; Liotta & Petricoin, 2000; Morris & Carey, 2007). These types of techniques are not without their disadvantages; they do not necessarily take into account any post-translational modifications of proteins; these can have significant effect on the function of the protein. Molecular profile techniques also do not take into account any mechanisms for over-expression of a gene, for example, gene amplification or epigenetic changes; tumour heterogeneity can also be overlooked (Morris & Carey, 2007). Despite these disadvantages molecular profiling is an extremely useful tool and has resulted in new ways of

classifying breast cancer. The different molecular subtypes of breast cancer reflect distinct gene patterns. Luminal subtypes of breast cancer tend to express hormone receptors and have properties of luminal epithelial cells such as expression of low molecular weight cytokeratins; ERBB2+ breast cancer over-express HER2 and 'normal breast' cancers were found to express genes associated with adipose tissue and non-epithelial cells such as lipoprotein lipase and integrin-α7 (Perou *et al.*, 2000; Sorlie *et al.*, 2001). Basal like breast cancer (BLBC) is so named due to expression of genes typical of basal/myoepithelial cells, such as high molecular weight cytokeratins (CK) demonstrated by expression arrays (Perou *et al.,* 2000; Sorlie *et al*., 2001; Rakha *et al.*, 2008). The consensus is that these types of breast cancer originate from the myoepithelial or basal layer of cells within the duct. Cytokeratins (CKs) are intermediate filament proteins that help comprise the mammalian cell cytoskeleton. The expression of particular CKs reflects the type, differentiation state and function of the epithelial cell. The expression of CKs can aid the classification of breast cancer (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Abd El-Rehim *et al.*, 2004; Nielsen *et al.*, 2004; Rakha *et al.*, 2006). For example, luminal epithelial cells, and therefore breast cancers of luminal type, typically express low molecular weight CKs such as CK7, CK8, CK18 and CK19; whilst myoepithelial or basal cells tend to express high molecular weight CKs such as CK5/6, CK14 and CK17 (Abd El-Rehim *et al.*, 2004; Rakha *et al.*, 2008). However, there is a debate as to whether BLBC actually originate from the myoepithelial/basal cells or just share a similar phenotype (Livasy *et al.*, 2006). Since molecular profiling is not currently cost efficient and feasible to carry out on a large scale, in particular on routine surgical specimens, IHC staining of the basal CKs such as CK5/6 CK14 and CK17 has been found to be an effective alternative to identify BLBC (El-Rehim *et al*., 2004; Nielsen *et al*., 2004; Reis-Filho & Tutt, 2008; Kuroda *et al*., 2009). From molecular profiling and IHC studies there is an overlap between TN and BLBC; 56-84% of TN cases express basal markers however not all BLBCs are TN (Perou *et al.,* 2000; Sorlie *et al*., 2001; Rakha *et al*., 2007; Rakha et al., 2008; Reis-Filho & Tutt, 2008). BLBC also tend to be associated with poorer survival (figure 1.10). Rakha *et al,* (2006) reported similar findings; BLBC

was associated with the poorest survival rates in both lymph node negative and positive cases. The development of BLBC has been associated with certain risk factors; for example, early menopause and multi-parity and not breast feeding; similarly to TN breast cancer, breast cancer at a younger age was also more likely to be BLBC (Millikan *et al.*, 2008).

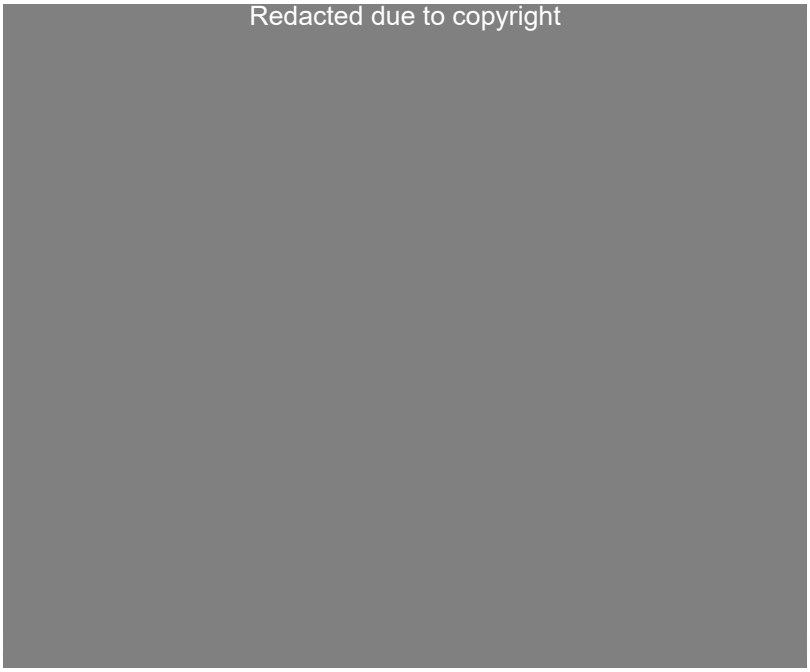


Figure 1.10 Survival probabilities of the molecular subtypes of breast cancer

Molecular profiling has led to the definition of 6 subtypes of breast cancer; luminal A, luminal B, luminal C, normal breast-like, ERBB2+ (Her2+) and basal like. Basal-like breast cancers have the poorest survival probability. Adapted from Sorlie *et al.* (2001).

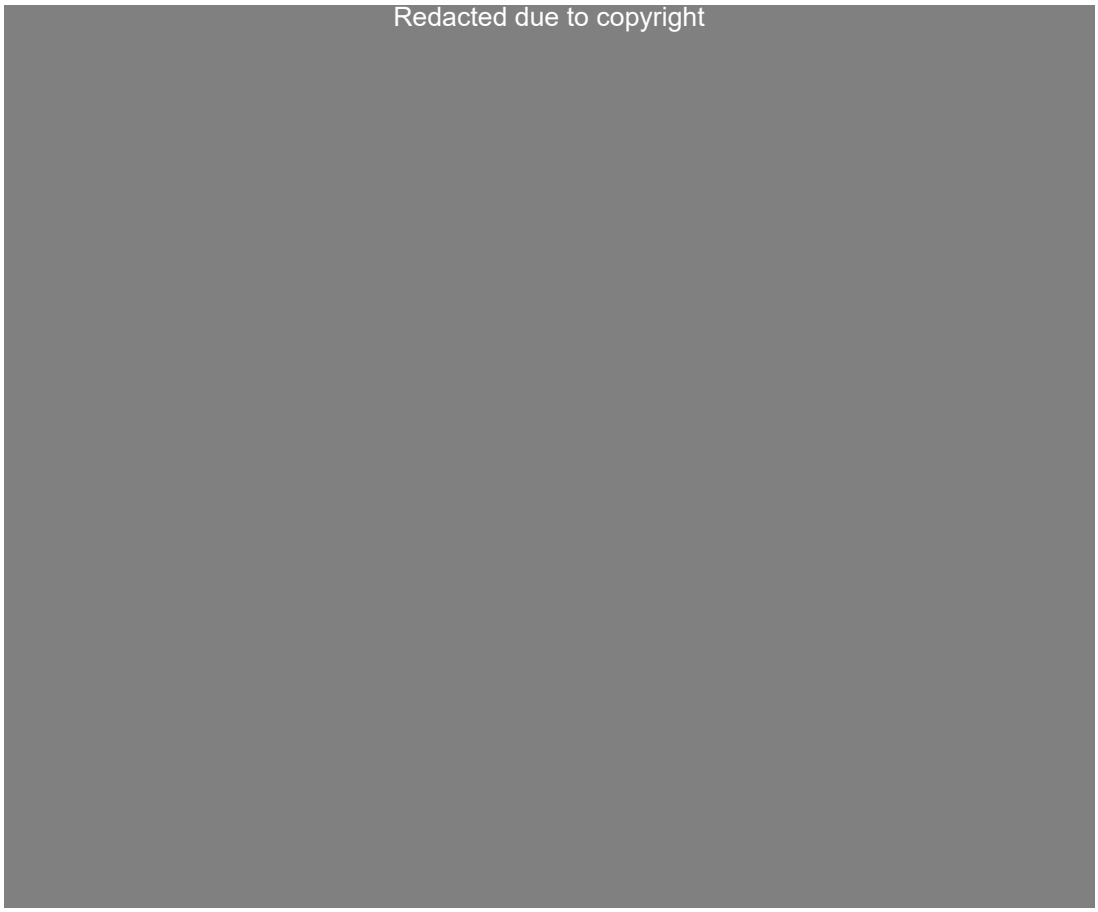
Molecular profiling has identified biomarkers associated with BLBC; for instance expression of *HER1/*EGFR in approximately 50% of cases, mutation of the *P53* gene and expression of the *C-KIT* gene (Rakha *et al*., 2006; Rakha *et al*., 2007; Kreike *et al*., 2007). There is also overlap between breast cancers with *BRCA1* mutations, sometimes called hereditary or familial breast cancer, with BLBC and TN breast cancer; nearly all *BRCA1* cases are of basal like phenotype and more than 60% of medullary and metastatic (breast cancers that have spread in to tissues beyond the breast)

BLBCs have *BRCA1* gene promoter methylation (Lakhani *et al*., 2005; Turner & Reis-Filho, 2006; Turner *et al.,* 2007; Reis-Filho & Tutt, 2008; Saal *et al*., 2008). Not all BLBC express these markers or have such genetic alterations. To define TN and BLBCs by the markers they do express would be more useful; particularly if there are targeted therapies available for those markers or related pathways.

1.5 Metabolism and candidate biomarkers in triple negative breast cancer

Obesity is on the increase worldwide particularly in western countries such as the UK and USA and it is estimated to increase healthcare costs by around £2 billion by 2030 in the UK (Wang *et al.*, 2011) due to the increased risk of developing diseases such as diabetes, heart disease and cancer. Postmenopausal women with a body mass index (BMI) of over 30 have a 31% increased risk of developing breast cancer compared to postmenopausal women with a BMI below 25; obese women who develop breast cancer also tend to have poorer overall survival and increased breast cancer progression and metastasis than women of a healthy weight (van den Brandt *et al.*, 2000; Morimoto *et al.*, 2002; Calle *et al.*, 2003; Lahmann *et al.*, 2004; Berclaz, *et al.*, 2004; Lorincz & Sukumar, 2006). One study reviewed the evidence behind the link between obesity and breast cancer and proposed the involvement of several pathways (figure 1.11). Increased fat levels or adiposity can increase circulating levels of insulin and insulin-like growth factor-I (IGF-I to be discussed further), tumour necrosis factor-α (TNF-α) and adipokines such as leptin (Lorincz & Sukumar, 2006). TNF-α is an inflammatory cytokine that is secreted by adipocytes that can lead to increased expression of aromatase, thus activating hormonal dependent tumourigenic pathways. Leptin is also secreted by adipocytes and regulates food intake by acting on the hypothalamus. Leptin can act as a growth factor in breast cancer cell lines, resulting in a more aggressive phenotype (Hu *et al*., 2002). Lorincz & Sukumar, (2006) used the evidence to propose the mechanisms by which obesity is linked to breast cancer; however the majority of mechanisms

are proposed to occur through the ER, this does not take into account the link between obesity and breast cancers that do not express ER such as many Her2 positive and TN breast cancers.



Lorincz A M , and Sukumar S Endocr Relat Cancer 2006;13:279-292

Figure 1.11 The proposed link between obesity and breast cancer

Adiposity can result in increased levels of insulin, IGF-I and TNF-α and leptin. IGF-I activates the IGF-1 receptor (IGF-1R) leading to phosphorylation of IRS and SHC. IRS-1, IRS-2 and SHC activate the PI3K and MAPK signalling pathways leading to increased cell proliferations. Adiposity leads to increased ER signalling through a number ways. For instance IGF-I can interact with oestradiol to increase cell proliferation by activation of the ER and IGF-1R. The high levels of the inflammatory cytokine TNF-α and the growth factor leptin in adiposity lead to increased aromatase activity and thus oestrogen synthesis. Consequently ER signalling pathways are activated that result in increased cell growth and invasion in breast cancer. This diagram is from Lorincz & Sukumar (2006).

Definitions: E2, oestradiol; E1, oestrone; T, testosterone; Δ4A, Δ4A-androstenedione; ApN, adiponectin; PI3K, phosphoinositide 3-kinase; IRS, Insulin receptor substrate; SHC, Src homology 2 domain containing transforming protein 1.

Type 2 diabetes is also associated with an increased breast cancer risk; insulin resistance leads to increased levels of circulating insulin that can activate signalling pathways such as RAS and *PI3K* (to be discussed further), through the insulin receptor and cause increased proliferation and decreased apoptosis (Wolf *et al.*, 2005).

There is evidence linking together aspects of metabolism and TN breast cancer, these are outlined in detail below and in figure 1.12. For instance, metabolic syndrome is a combination of medical disorders such as central obesity, raised triglyceride levels in the blood and high blood pressure, which substantially increase an individual's risk of developing heart problems or diabetes. Investigation has taken place linking breast cancer with the metabolic syndrome and its associated diseases; metabolic syndrome is more common in patients with TN breast cancers than other breast cancers but it is yet to be determined whether metabolic syndrome increases the risk of TN disease (Maiti *et al.*, 2010). There is also conflicting evidence as to whether obesity increases the risk of developing TN breast cancer; one study found that a high BMI increases the risk of developing TN breast cancer (Pierobon & Frankenfeld, 2012), in contrast to this another found that there was no difference between obesity and physical activity and the subtype of breast cancer (Phipps *et al.*, 2011). Breast cancers with *BRCA1* abnormalities tend to be of TN/basal like phenotype; Brca1 has recently been shown to be involved in the regulation of fatty acid synthesis through interaction with acetyl-coA carboxylase (ACC) (Brunet *et al.*, 2008). Brca1 binds to the phosphorylated or inactive form of ACC, the rate limiting enzyme in fatty acid synthesis, and stabilises it. If Brca1 is mutated or present in low levels, ACC is more abundant in its active form, thus converting acetyl coA to malonyl coA to increase fatty acid synthesis by fatty acid synthase (FAS) (section 1.3.5). It has been suggested that women with *BRCA1* mutations could be treated with drugs used for diabetes such as metformin or through calorie deprivation and exercise, as this would increase AMP-activated protein kinase (AMPK) levels that would in turn inhibit ACC (Magnard *et al.*, 2002). Fatty acid synthase (FAS) is another biomarker linking metabolism and cancer; FAS is up-regulated in many cancers including those of the breast,

prostate, colon and endometrium but it is not fully understood why or how (Kuhajda, 2000). FAS expression was associated with an increased risk of breast cancer reoccurrence (Alo' *et al.*, 1996) and up-regulation of FAS confers chemotherapy resistance; down regulation of FAS caused breast cancer cell lines to become more sensitive to chemotherapy drugs (Liu *et al.*, 2008; Zeng *et al.*, 2010). Insulin-like growth factor (IGF) -I has been shown to up-regulate FAS in malignant breast cancer cells and when FAS was suppressed, IGF-I mediated cell growth was inhibited and the cells were no longer resistant to palmitate induced death (Zeng *et al.*, 2010). Figure 1.12 shows the interaction of breast cancer biomarkers and aspects of metabolic pathways. For instance liver kinase B1 (*LKB1)* is a tumour suppressor gene that can phosphorylate AMPK to activate it; this prevents fatty acid synthesis (Shaw *et al.*, 2004). The biomarkers of interest in the present study that will be discussed further are fatty acid binding protein 7 (FABP7), insulin-like growth factor binding protein 2 (IGFBP-2) and phosphatase and tensin homolog (PTEN).

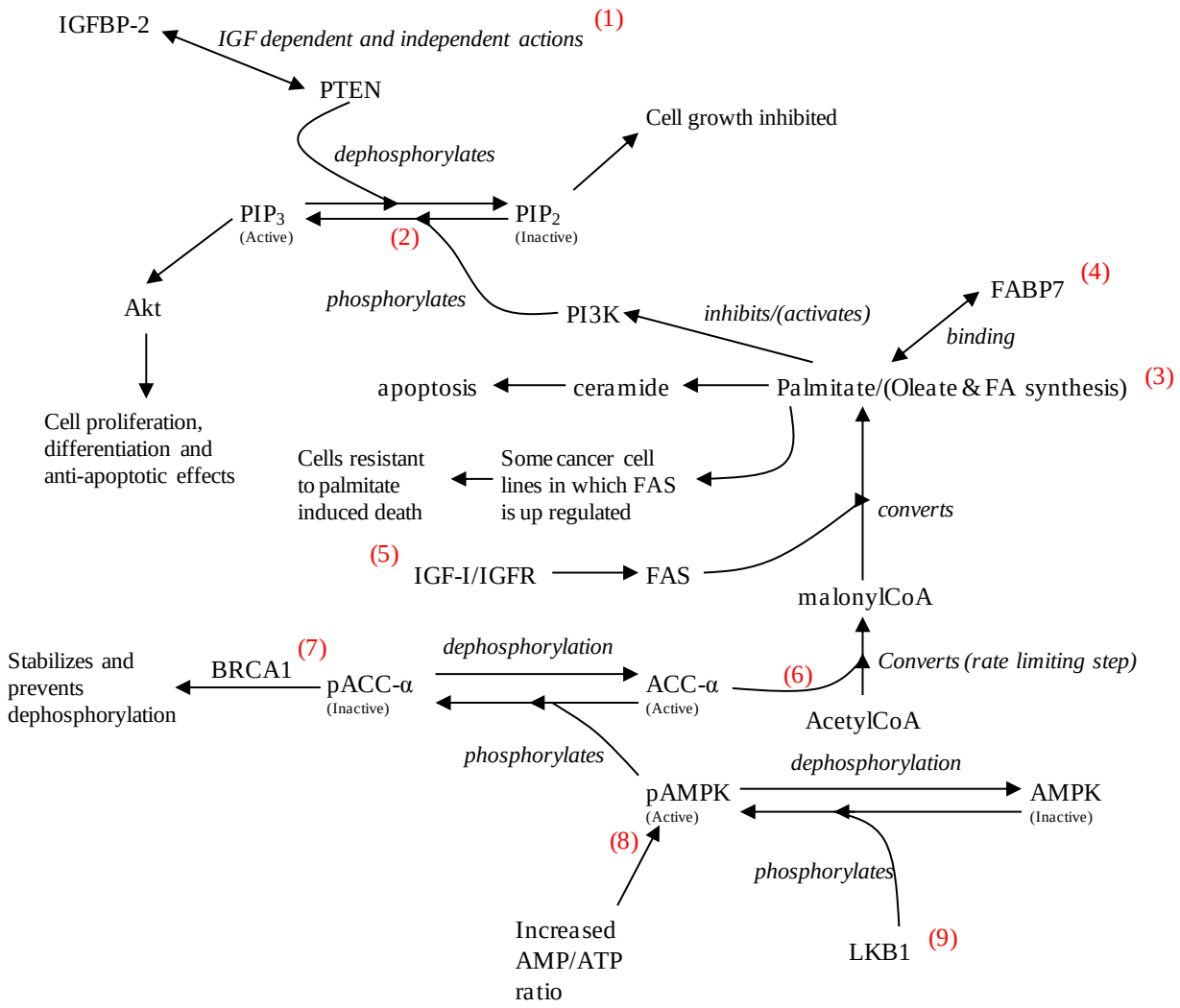


Figure 1.12 The interaction of metabolic pathways and breast cancer

The interaction of PTEN and IGFBP-2 is outlined further in section 1.7.1 (1). The *PI3K* pathway phosphorylates PIP_2 to PIP_3; PIP_3 is an important activator of the Akt pathway, which impacts on cell growth and proliferation. PTEN dephosphorylates PIP_3 to the inactive form PIP_2 so the Akt pathway is not activated and thus cell proliferation is inhibited (Panigrahi *et al.*, 2004) (2). It has been found that in the breast cancer cell line MDA-MB-231 palmitate inhibits the *PI3K* pathway so in turn the Akt is inhibited; oleate activates the *PI3K* pathway in turn promoting cell proliferation (Hardy *et al.*, 2000). Palmitate can induce apoptosis by its conversion to ceramide and also independently of ceramide conversion (de Pablo *et al.*, 1999; Listenberger *et al.*, 2001). When FAS is up-regulated some breast cancer cells are resistant to palmitate induced apoptosis (Zeng *et al.*, 2010) (3). FABP7 binds to FAs such as palmitate and oleate to transport them and regulate FA metabolism (4). The binding of IGF-I to the IGF-receptor (IGFR) has been shown to up-regulate the expression of FAS (Zeng *et al.*, 2010) (5).Acetyl-CoA Carboxylase-α (ACC- α) is the rate limiting step in FA synthesis, it converts AcetylCoA to MalonylCoA. One study found that by inhibiting ACC- α apoptosis of cancer cells was induced (Chajes *et al.*, 2006) (6). When ACC- α is phosphorylated (pACC- α) it become inactive. Studies have shown that Brca1 binds to pACC- α and stabilizes it; therefore in cancers with *BRCA1* mutations, Brca1 can no longer bind to pACC- α and stabilize the inactive form (Brunet *et al.*, 2008; Magnard *et al.*, 2002; Moreau *et al.*, 2006). This means that there is more active ACC- α and thus more conversion of AcetylCoA to MalonylCoA for FA synthesis (7). Phosphorylated AMPK (pAMPK) is responsible for phosphorylating ACC- α to inactivate it. Under low energy conditions there is an increased AMP: ATP ratio; AMPK responds to this by preventing fatty acid synthesis and thus prevents the consumption of ATP until the balance is corrected (Shaw, 2006). One study, immunohistochemically staining for pAMPK, found evidence to suggest that AMPK can be dysfunctional in primary breast cancer and thus reactivation of the AMPK pathway may be of therapeutic benefit (Hadad *et al.*, 2009) (8). LKB1 is a tumour suppressor gene. LKB1 has been found to phosphorylate AMPK activating it and this preventing fatty acid synthesis (Shaw *et al.*, 2004) (9).

1.6 Fatty acid binding protein 7

Fatty acid binding protein 7 (FABP7) is involved in aspects of metabolism and may be a useful biomarker in the investigation of TN breast cancer. FABP7 is also known as brain lipid binding-protein (BLBP/FABP-B) as it was first discovered in the brain. There are 9 FABPs, all around 15kDa in size, that have a role in the solubilisation of various fatty acids (FAs) and their transport, storage and FA metabolism. The exact function and mechanisms of action of FABPs are not fully understood, however much work has been carried out examining their structure in order to uncover their function. For example FABPs have a β-barrel tertiary structure that forms a cavity where FAs bind; additionally FABPs also have a helix-loop-helix motif which is thought is the point of entry for the FAs. Gene expression analysis has revealed that FABP7 is over expressed in BLBC (Sorlie *et al.*, 2001). IHC has also demonstrated that FABP7 is associated with BLBC and TN breast cancer though there is conflicting evidence as to whether FABP7 is associated with poor or good prognosis; one breast cancer study using IHC concluded that FABP7 was associated with improved survival probability (Zhang *et al.*, 2010); another concluded that FABP7 was associated with poor survival (Tang *et al.*, 2010). Such differences could be a reflection of the cohorts being unselected and different in size (Zhang *et al.*, 2010; Tang *et al.*, 2010). Studies have shown that FABP7 inhibits cancer cell growth; over expressing FABP7 in the MDA-MB-231 breast cancer cell line significantly inhibited cell growth and promoted differentiation (Shi *et al.*, 1997). This effect was synergised with docosahexaenoic acid (DHA) supplementation (Wang *et al.*, 2000). In contrast a study on FABP7 in melanoma found that down-regulating FABP7 expression decreased cell proliferation and invasion and when it was over-expressed, cell proliferation and invasion were increased (Goto *et al.*, 2006). A continuation of this demonstrated, using IHC, that expression of FABP7 was associated with poorer survival and relapse of melanoma, although FABP7 expression was lost when patients progressed from primary melanoma to metastatic melanomas (Goto *et al.*, 2010). Loss of heterozygosity of the FABP7 gene in metastatic melanomas may be responsible for the loss of FABP7 expression and thus improved prognosis (Goto *et al.*, 2010). In glioblastomas

FABP7 is also associated with poor prognosis and dietary supplementation of DHA has been found to increase FABP7 mRNA in rat brain (Kaloshi *et al.*, 2007; Nasrollahzadeh *et al.*, 2008). These studies demonstrate the possibility of using FABP7 as a biomarker of prognosis in TN breast cancer. A recent study by Mita *et al.* (2010) indicated that it was the relative abundance of FAs that influenced the role of FABP7 in gliomas. Figure 1.13 illustrates how the relative abundance of arachidonic acid (AA) and DHA alter how FABP7 aids induction or inhibition of pro-migratory genes through activation of cyclooxygenase 2 (COX2) and PPAR-γ pathways. Such results may link in with indications that the binding of a FABP to a FA induces a conformational change in the FABP that enhances or decreases its ability to bind and interact with other proteins and thus affect its function (Storch & McDermott, 2009). Additionally the relative expression of FABP7 with COX2 or PPAR-γ could be predictive of prognosis.

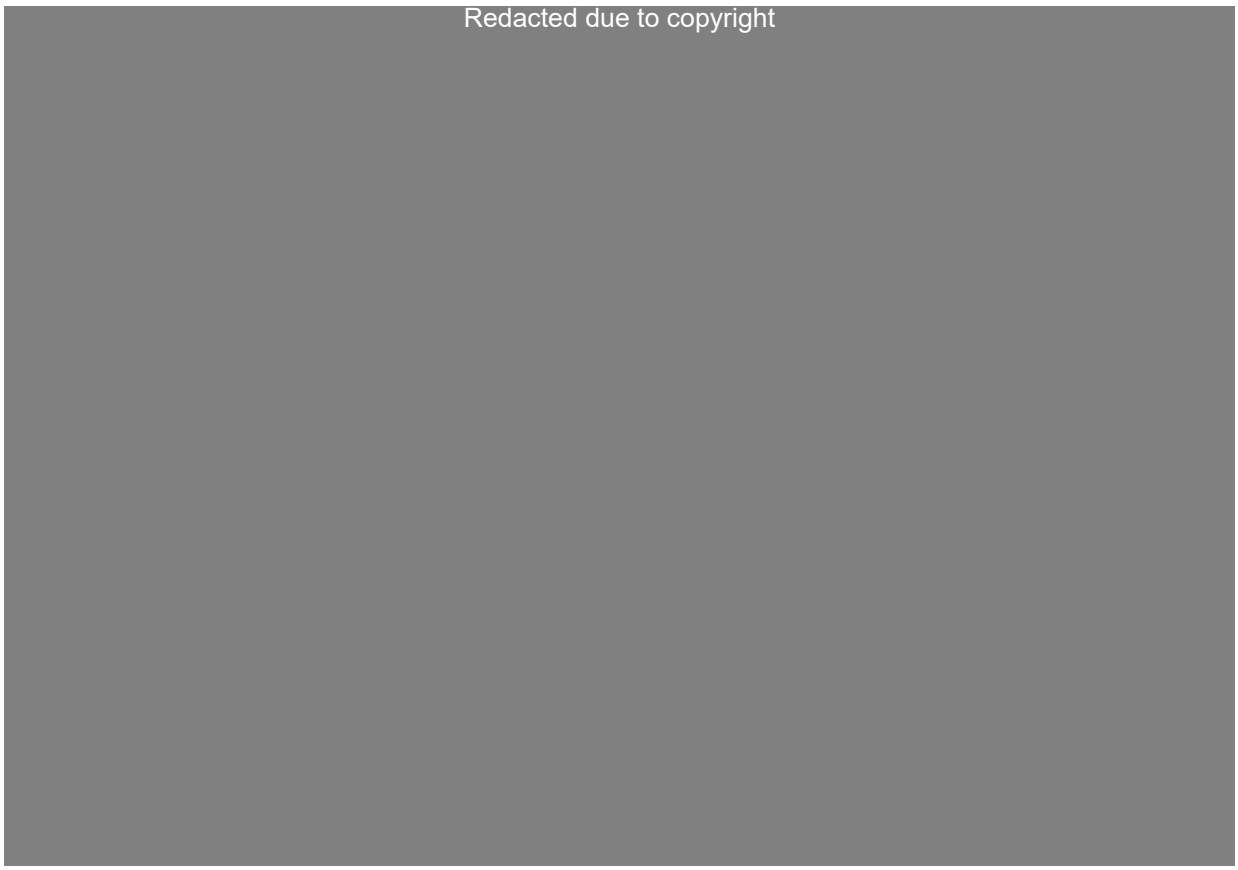


Figure 1.13 Cytoplasmic and nuclear roles of FABP7 and its interaction with AA and DHA

FABP7 can bind to AA or DHA depending on the relative abundance of each that has entered or has been produced in the cell. FABP7 can shuttle AA to the COX2 pathway. The COX2 pathway leads to the conversion of AA to prostaglandin (PGE2). PGE2 has many functions including roles in inflammation; in this instance PGE2 functions to activate downstream signalling pathways and induce migration and proliferation. FABP7 can also shuttle DHA to the nucleus where DHA is transferred to PPAR-γ. DHA and PPAR-γ bind to DNA and inhibit COX2 pathway activation and thus PGE2 synthesis leading to down-regulation of pro-migratory signalling. The binding of DHA and PPAR-γ and DHA to DNA can also lead to activation of anti-proliferative pathways. Diagram adapted from (Mita *et al.*, 2010).

FABP3, also called mammary derived growth inhibitor (MDGI), has high sequence homology with FABP7 and has been shown to be down-regulated by IGF-I dose dependently in mutant mice. Furthermore it was found that insulin like growth factor-II (IGF-II), a ligand of the IGF axis, expression was inversely correlated with FABP3 expression (Huynh & Beamer, 1998). FABP3, similarly to FABP7, is also thought to inhibit cancer cell growth and promote differentiation; one study found this by expressing a peptide designed to mimic the effects of FABP3 in breast cancer cell lines (Wang *et al.*, 2000). Nevo *et al.* (2010) found that one possible reason for the tumour suppressing activity of FABP3 is that it regulates integrin activity which in turn has a role in cell motility, growth and adhesion. Considering the sequence homology of FABP3 and FABP7 it may be possible that FABP7 also has interactions with integrin, IGF-I and –II and thus the IGF axis.

This evidence suggests that the role of FABP7 may be different in different cancers and that expression may relate to cancer progression and stage. However, the association of FABP7 and prognosis in breast cancer is not understood; learning more about it will add to understanding about what drives TNBC. Equally as important as the function of FABP7 are the mechanisms that regulate it.

1.6.1 Methylation and acetylation of FABP7

Epigenetic changes alter gene expression without affecting the sequence of DNA (Esteller, 2008); such changes have been shown to play an important role in gene expression of cancer cells and thus cancer development and progression.

Two examples of epigenetic mechanisms are histone acetylation and DNA methylation. Two families of enzymes regulate histone acetylation, histone acetyl transferases (HATs) and histone deacetylases (HDACs). HATs weaken the interactions between DNA and histones by acetylating lysine residues; the weakened interactions cause the chromatin to decondense and allow

transcription of genes to occur (Hassig & Schreiber, 1997; Struhl, 1998; Wade *et al.*, 1997). HDACs reverse the acetylation so chromatin condenses and thus gene transcription is prevented (Struhl, 1998; Wade *et al.*, 1997). DNA methylation is the addition of a methyl group to CpG regions of DNA. CpG regions are composed of a cytosine base linked to a guanine base by a phosphodiester bond. DNA methyltransferases (DNMT) methylate the cytosine to make 5-methylcytosine; which is important in determining whether a gene is expressed (Pradhan & Esteve, 2003). If methylation occurs in the promoter region of a gene then that gene can be silenced because binding of transcriptional proteins is impeded (Momparler & Bovenzi, 2000; Worm & Guldberg, 2002). DNA methylation can also silence genes through facilitation of histone deacetylation. Methyl-CpG binding proteins (MeCPs) are recruited to the methylated sites and can bind to DNA and chromatin (Nan *et al.*, 1997). The MeCPs then in turn recruit HDACs that, as described, remove acetyl groups and cause chromatin to condense (Nan *et al.*, 1998). Additionally repression of transcription can occur through the binding of HDACs directly to the DNA methyltransferase DNMT1 (Fuks *et al.*, 2000). In cancer these mechanisms can become dysregulated leading to hypermethylation or hypoacetylation so a gene is under-expressed or hypomethylation or hyperacetylation where a gene is over-expressed (Archer & Hodint, 1999; Grønbaek *et al.*, 2007). Epigenetic changes have been studied within different subtypes of breast cancer to try to discover the difference in what drives them and to open up new targets for treatment. Suzuki *et al*. (2009) used immunohistochemistry (IHC) to show decreased histone acetylation in DCIS and invasive ductal carcinoma (IDC) compared to normal breast epithelium; they also found that HDAC expression was also decreased in tumours compared to normal tissue. It was suggested that this may be due to the relative activity levels of HDACs and HATs. Elsheikh *et al*. (2009) reported similar findings also using IHC. Differing methylation states have been found between the different subtypes of breast cancer; in particular it was noted that luminal B breast cancers have a high global methylation frequency compared to other subtypes (Holm *et al.*, 2010). A recent study identified novel genes and their methylation status and found this was associated with

prognostic factors in breast cancer; in particular a gene called *RECK* was associated with poorer prognosis when it was methylated (Hill *et al.*, 2011). There is some evidence suggesting that *FABP7* may be epigenetically changed in cancer. For example differences in *FABP7* methylation was observed between subsets of chronic lymphocytic leukaemia; *FABP7* was listed as a methylated gene in the immunoglobulin heavy-chain variable mutated genes type (Kanduri *et al.*, 2010). Etcheverry *et al*. (2010) used techniques to study whole genome methylation and identified *FABP7* as one of several genes that were hypomethylated in glioblastomas compared to the normal brain; this results in over-expression of FABP7 protein in glioblastomas. As discussed FABP3 has a similar structure and function to FABP7 in breast cells. A study by Huynh *et al*. (1996) demonstrated that *FABP3* is hypermethylated and thus silenced in both breast cancer cell lines and a selection of patient breast tumours. It is therefore possible that *FABP7* may be hypermethylated in some breast cancers. Currently there is little information regarding changes in the acetylation status of FABPs in cancer. However it has been reported that the expression of FABPs can be altered through changes in histone acetylation; trichostatin A (TSA), an HDAC inhibitor, was demonstrated to increase *A-FABP* (*FABP4*) expression in RAW 264.7 macrophages (Coleman *et al.*, 2010). Treatment with TSA was also enough to negate the inhibitory effect of linoleic acid on *FABP4* expression (Coleman *et al.*, 2010). It would be useful and interesting to see if FABP7 in breast cancer cell lines is regulated by epigenetic mechanisms.

1.6.2 Fatty acid regulation of FABP7

There are many other mechanisms other than acetylation and methylation that can alter gene expression. For example, there is an abundance of evidence demonstrating that fatty acids (FAs) can alter gene expression. FAs have important roles in metabolism, cell signalling and maintenance of membrane fluidity and permeability; they are vital components of living cells. As reviewed by Duplus *et al*., (2000), FAs can negatively and positively regulate gene expression; this

can be direct, for example, by the binding of FAs to transcription factors, or indirect by the regulation of signal transduction pathways (figure 1.14) (Jump, 2004). Gottlicher *et al*., (1992) were among the first to determine that fatty acids such as linoleic acid and oleate can bind to nuclear receptors such as PPARs; in turn affecting gene transcription. Other transcription factors targeted by FAs include hepatic nuclear factor 4 (HNF4), retinoic X receptor (RXR) and sterol regulatory element binding proteins (SREBPs) (Jump & Clarke, 1999; Duplus &Forest, 2002; Jump, 2004; Sampath & Ntambi, 2004;). FAs not only alter gene expression at a transcriptional level but also at a post-transcriptional level; for example through the regulation of mRNA degradation (Duplus & Forest, 2002).

Figure 1.14 The mechanisms by which fatty acids can alter gene expression.

Fatty acids can directly alter gene expression by binding to a transcription factor. Fatty acids can also change gene expression indirectly by modifying membrane composition and cellular metabolism; this in turn activates signalling pathways which can regulate the abundance and activity of transcription factors. Ultimately changes in the gene expression affect cellular metabolism, growth and differentiation. This diagram is taken from Jump (2004).

Lipid binding studies have shown that some FABPs have an affinity for certain FAs; this could help explain FABP functions although evidence is conflicting. For instance Zimmerman *et al.* (2001) noticed that generally FABPs have a higher affinity for unsaturated FAs. In contrast to this, it was confirmed that although FABP7 has a high affinity for unsaturated FAs it actually bound saturated FAs with a higher affinity; this was the opposite of what was predicted. Since FABP7 is found in the brain and as the brain requires high levels of unsaturated FAs, it was thought that FABP7 would have a higher affinity for unsaturated FAs (Richieri *et al.*, 2000). Differences between species origin of FABP and the types of FA they bind have also been found, for example, murine FABP7 does not bind to palmitate but human FABP7 binds to palmitate, oleate, docosahexaeonic acid and arachidonic acid (Xu *et al.*, 1996; Richieri *et al.*, 2000; Zimmerman *et al.*, 2001).

FAs alter the expression of FABPs; for example FABP4 levels were altered in RAW264.7 macrophages by exposing the cells to FAs (Coleman *et al.*, 2010). Unsaturated FAs such as linolenic acid and palmitoleic acid decreased FABP4 mRNA and protein; however the unsaturated FA eicosapentaenoic acid significantly decreased FABP4 protein but mRNA levels were not notably different from the control (Coleman *et al.*, 2010). Hyder *et al.* (2010) found that FABP3 and FABP5 mRNA and protein increased in rat pancreatic islet cells and NS-1E cells (a rat pancreatic β-cell line) by treatment with palmitate and oleate; β-oxidation of these FAs is required for the increased expression. This was elucidated by treating the cells with an inhibitor of carnitine palmitoyl transferase 1, an enzyme catalysing the rate limiting step in β-oxidation (Hyder *et al.*, 2010). Interestingly the research also found that agonists and antagonists of PPARs altered the expression of both FABPs; of note both FABP3 and 5 mRNA were increased by a PPAR-α agonist in addition FABP5 mRNA was also increased by a PPAR-γ agonist (Hyder *et al.*, 2010). This indicates that the FAs interact with PPARs to increase FABP expression. FABP1, also known as liver-FABP, was up-regulated at both the mRNA and protein level by oleate in rat hepatoma cells (Meunier-Durmort *et al.*, 1996). It was found that mRNA levels increased in a dose dependent manner and that the protein increase relied on *de novo* protein synthesis demonstrated using the protein

synthesis inhibitor cycloheximidine (Meunier-Durmort *et al.*, 1996). Other long chain FAs found to increase *FABP1* expression in the rat hepatoma cells included palmitate, AA and linoleic acid. The group went on to show the up-regulation of FABP1 mRNA was potentiated by treatment with 9-cis-retanoic acid (9cis-A) (Poirier *et al.*, 1997). 9cis-RA binds to RXRs and oleate bind to PPARs; it was postulated that a heterodimer of RXR and a PPAR bound to a PPAR response element (PPRE) in the *FABP1* gene initiating transcription (Poirier *et al.*, 1997). Another instance of FA operating with other molecules to alter expression is shown by DHA activating the RXR by acting as a transcription factor when bound to FABP7; however this causes the activation of the RXR rather than *FABP7* but it is plausible that there may be some feedback mechanism involved that in turn alters the expression of FABP7 (Liu *et al.*, 2012). FABP3 has been shown to have similarities in the way it acts to FABP7, particularly in breast tissue. FABP3, also called heart-FABP, expression can be increased at the mRNA level in cardiomyocytes by treatment with long chain FAs (Chang *et al.*, 2001). FAs including palmitate, oleate and AA increased FABP3 mRNA at least 2 fold after 10 hours of incubation, it was reported that levels remained high after 24 hours but cell viability drastically decreased so incubation remained a maximum of 10 hours (Chang *et al.*, 2001). Palmitate produced the greatest increase of mRNA and it was postulated that this was due to accumulation of palmitate in the cytoplasm inhibiting fatty acid oxidation(Chang *et al.*, 2001). These pieces of evidence demonstrate that the relationships between FAs and FABP gene expression are complex; as mentioned previously FA can alter gene expression directly or indirectly and pre- or post-transcriptionally. Any of these processes are possible in the FA regulation of FABP expression.

Considering the evidence that FAs can alter the expression of FABPs and that FABP7 is associated with TN breast cancer as well as regulating FA metabolism, it is logical that FAs could alter the expression of FABP7 in TN breast cancer.

1.7 Insulin-Like Growth Factor Binding Protein 2

Insulin-Like Growth Factor Binding Protein-2 (IGFBP-2) is a member of the Insulin-like growth factor family of which there are 6 binding proteins and a type 1 IGF receptor (IGF-IR) that regulate the mitogenic and anti-apoptotic effects of two ligands, insulin-like growth factor –I and –II (IGF-I and IGF-II). IGFs, particularly IGF-II, have been found to be essential in foetal growth (Duan, 2002); links with the growth hormone axis and epidemiological studies have shown that IGFs are important in determining height; individuals who are taller are more at risk of developing cancer particularly of the breast or prostate (Okasha *et al.*, 2002). Moreover circulating IGFs have been linked to the risk of developing breast cancer; for instance one study found that increased serum IGFs were linked to an increased risk of developing breast cancer in premenopausal women but not those who were postmenopausal (Hankinson *et al.*, 1998). Another study had similar findings and also noted that low serum IGFBP-3 levels were associated with an increased risk of developing breast cancer in premenopausal women (Allen *et al.*, 2005). Both IGF and IGFBP expression fluctuate throughout mammary gland development and lactation; altering the expression of members of the IGF axis impacts on mammary gland growth and differentiation (Flint *et al.*, 2008). One study, determining IGFBP-2 expression with IHC, found that the more aggressive the breast cancer the higher the IGFBP-2 expression when compared to benign lesions (Busund *et al.*, 2005). IGFBP-2 has also been demonstrated to be over expressed in anti-oestrogen resistant breast cancer cell lines (Maxwell & van den Berg, 1999; Juncker-Jensen *et al.*, 2006). So *et al*. (2008), demonstrated that IGFBP-2 was not of prognostic value in hormone receptor positive breast cancers and that there was no correlation with Her2; moreover in the hormone receptor negative cases IGFBP-2 was associated with poorer disease specific survival. It was originally thought that the actions of IGFBP-2 and the other IGFBPs were dependent on IGFs but IGF independent actions have been identified. For instance, some IGFBPs have heparin binding sites (Firth & Baxter, 2002). It has recently been found that IGFBP-2 is a novel regulator of the tumour suppressor Phosphatase and Tensin-homolog (PTEN) (section 1.8). Perks *et al*., (2007)

illustrated that the breast cancer cell line MCF-7 does not respond to high levels of IGF-II as a result of PTEN induction and that IGFBP-2 suppresses PTEN when it is not bound to IGF-II as shown in figure 1.15. Alternatively, PTEN may regulate the expression of IGFBP-2 as demonstrated by Levitt *et al*., (2005) in the U251 glioma cell line; inducing PTEN expression decreased the expression of IGFBP-2

Figure 1.15 The IGF independent actions of IGFBP-2 on PTEN

When IGF-II binds to the IGF-I receptor there is a feedback increase in PTEN to prevent excessive proliferation. However, when IGFBP-2 is free from IGF-II it can bind to an Integrin receptor and down-regulate PTEN. Hence when IGFBP-2 is over expressed the feedback increase in PTEN in response to IGF-II is blocked. This diagram is from Perks *et al*. (2007).

Considering the involvement of IGFBP-2 with metabolic pathways and the interaction with PTEN, both IGFBP-2 and PTEN are relevant and important to study in relation to TN breast cancer.

1.8 Phosphatase and tensin homolog

Phosphatase and Tensin-homolog (PTEN) is a tumour suppressor gene found on chromosome 10 (Di Cristofano & Pandolfi, 2000). Its absence or mutations are implicated in many cancers including those of the breast, prostate and lung (Li *et al.*, 1997; Torres *et al.*, 2001). PTEN works by dephosphorylating PIP_3, a product of the *PI3K* pathway, to PIP_2 so the Akt signalling pathway is no longer activated and thus cell growth is inhibited and apoptosis is promoted (Salmena *et al.*, 2008); figure 1.12 shows how PTEN is involved in aspects of metabolism. PTEN has also been found to have an important role in DNA repair. PTEN regulates the expression of RAD51; mutation or loss of PTEN results in a deficiency to repair DNA DSB through lack of complexing of RAD51 with Brca (figure 1.8) (Shen *et al.*, 2007). In unselected breast cancer cohorts with the expected frequency of Her2 and hormone receptor positive cases, PTEN loss appears to be a relatively rare event (Mendes-Pereira *et al.*, 2009) and the frequency of PTEN loss in breast cancer varies greatly depending on the study. One study has reported PTEN loss to be as high as 48% in a breast cancer cohort not selected on the basis of either hormone receptor or Her receptor status (Depowski *et al.*, 2001); although most other studies report a much lower incidence in cohorts of sporadic breast cancer; eight percent (Panigrahi *et al.*, 2004), fifteen percent (Perren *et al.*, 1999) and twenty-eight percent (Lopez-Knowles *et al.*, 2009). Theses disparities most likely reflect differences in the reliability of the antibodies used, the age of the formalin fixed blocks, the detection methods and the interpretation of the results, particularly with respect to the definition of PTEN loss. Conversely, a recent study by Lopez Knowles et al., (2010) would suggest that PTEN loss is a more frequent event in BLBC; with nineteen of twenty-nine cases showing loss of PTEN. In agreement with this Marty *et al*. (2008) found that PTEN loss and alterations were more common in BLBC than Her2 positive breast cancer. PARP inhibitors have been demonstrated to be effective in breast cancers with defective DNA repair however the trials did not show significance. It could be argued that future trials may provide more promising results if patients within the triple negative category are selected on the basis of assayable

defects in homologous repair. Relatively few patients would be selected if they were to be chosen purely on the basis of *BRCA* mutations alone (Young *et al.*, 2009). In addition, Brca analysis utilising immunohistochemistry has yet to be fully validated for its efficacy on routinely fixed and paraffin processed cases of triple negative breast cancer. However, in identifying markers predictive of likely response to PARP inhibitors cell line models have shown that tumour cells with PTEN loss are sensitive to PARP inhibition and the platinum salt, cisplatin (Mendes-Pereira *et al*., 2009), in a similar fashion to tumour cells carrying *BRCA* mutations. The initial studies suggested that homozygous PTEN mutations that truncate the open reading frame sensitizes tumour cells to PARP inhibitors, in a similar way as cancer cells with *BRCA1* and *BRCA2* deficiencies (Turner *et al.*, 2008; Farmer *et al.*, 2005). In addition reconstitution of the prostate cancer cell line PC3, known to be lacking in PTEN expression, with ectopic expression of a catalytically inactive PTEN allele, was sufficient to restore resistance to PARP inhibitors. It is envisaged that the role PTEN plays in determining response to PARP inhibitors, is mediated by nuclear activity of the protein and it has been proposed that a complete absence of nuclear PTEN expression, determined by immunohistochemistry could effectively predict response (Mendes-Pereira *et al.*, 2009). For these reasons PTEN is an interesting and highly relevant marker to investigate in TN breast cancer.

1.9 Understanding biomarkers in populations presenting with aggressive breast cancer

There is a general lack of standardised comparative studies between the types of breast cancer affecting women of European descent and those breast cancers affecting Asian women. Breast cancer in Malaysian women occurs less frequently than in Western women but tends to be of a more aggressive subtype and occurs at a younger age. In one study, 58% of patients had hormone receptor negative breast cancer (Agarwal *et al*., 2007), which is particularly aggressive, whilst in the West this is only approximately 20-25% (Rhodes *et al*. 2000). However one study reported an incidence of 17.6% TN breast cancer cases in Malaysia; it was also found that TN breast cancer was

associated with higher grade (Tan *et al*., 2009). This is in conjunction with other studies and makes TN breast cancer in Malaysia important to study. In the UK 81% of TN breast cancer cases occur in patients 50 years old or over whereas in Malaysia less than 50% occur in this age category and 60% of patients are premenopausal, whilst in the West the majority of breast cancers occur in post menopausal women (Yip *et al*., 2006; Yip 2009). There are also differences in the mean age of occurrence between the three main ethnic groups in Malaysia; in Malays it is 48.1 years, Chinese 51.4 years and Indians 52.3 years (Yip *et al*., 2006). There are several theories as to why there are differences between UK and Malaysian breast cancer. One is that populations tend to be younger in developing countries, hence why in Malaysia there are a higher proportion of younger women with breast cancer than in the UK (Yip *et al*., 2006). Another is that until relatively recently the lifestyle of Asian women has been less associated with known risk factors of breast cancer, compared to the lifestyle of Western women. In Asia women generally have less oestrogen exposure that women in the UK; they tend to start menstruating later and go through the menopause earlier; they tend to bear children younger and breastfeed for longer and the use of the contraceptive pill is very low (Agarwal *et al*., 2007). Lack of screening programs or reluctance to see a doctor due to cultural influences may account for the lower incidence and may also explain why Malaysian breast cancer results in higher mortality, as it is detected much later on (Agarwal *et al*., 2007; Yip 2009). There may also be differences in methodology in the assay and interpretation of hormone receptor status and Her2 status in Malaysia when compared to the UK; perhaps due to a lack of resources. Consumption of dairy products is much less in Malaysia than in the UK. High dairy product consumption has been associated with high serum levels of IGF-I and IGF-II. A study by Rinaldi *et al*., (2005) found that high IGF-I serum levels were associated with higher breast cancer risk particularly in younger women. In Chinese women high IGF-I serum levels were also found to be associated with increased breast cancer risk but only in premenopausal women (Yu *et al*., 2002). The differences between populations may be a result of dietary or oestrogen exposure factors or they may even be a result of genetic differences.

1.10 Study design

This study sought to investigate patterns of FABP7 expression in patient samples and cell lines, what regulates FABP7 expression in breast cancer cell lines, the effect of FABP7 over-expression on breast cancer cells and the patterns of expression of IGFBP-2 and PTEN in patient samples. This study design section brings together the various investigations in this thesis and justifies the choices of cell lines, cell line treatments and outlines the patient demographics. Table 1.3 gives the details of the breast cell lines used in this work.

Cell Line	Gene Cluster	ER	PR	HER2	Doubling Time (hr)
BT-20	Basal A	-	-	-	30
HS578T	Basal B	-	-	-	76
MCF-7	Luminal	+	+	-	22
MCF10a	Basal B	-	-	-	27
MDA-MB-231	Basal B	-	-	-	33
MDA-MB-361	Luminal	+	-	+	75
MDA-MB-453	Luminal	-	-	-	36
SKBR3	Luminal	-	-	+	26
T47D	Luminal	+	+	-	35

Table 1.3 The features of the cell lines used in this work

This table shows the ER, PR and HER2 status of the cell lines used in this work as well as the molecular subtype (gene cluster) and the observed doubling time.

1.10.1 Methylation and Acetylation studies

To understand if epigenetic mechanisms were involved with the regulation of FABP7 expression, *5-Aza-2*'-deoxycytidine a demethylating agent and Trichostatin A an HDAC inhibitor were used to treat breast cancer cell lines. BT-20 and MDA-MB-231 cell lines were selected for these treatments because they are of TN and basal-like phenotype (highly important features in the scope of this study) (table 1.3) and because work in chapter 3 demonstrated that expression of FABP7 mRNA and protein in these cell lines was higher compared to others tested. The doses of AZA and TSA were chosen based on pre-published dose responses (using breast cancer cell lines)

by collaborators at the University of Bristol. The duration of TSA treatment was kept at a maximum of 24 hours because preliminary work (not included) showed that extending it caused significant cell death. The maximum AZA treatment duration was 72 hours because beyond this preliminary work (not shown) showed extensive cell death. In addition considering the short half life of AZA it is unlikely increasing treatment duration would have impacted on FABP7 expression.

1.10.2 Fatty acid choice and dose justification

To study changes in FABP7 expression BT-20 and MDA-MB-231 cells were treated with the FAs oleate, palmitate, AA and DHA. The cell lines were selected for the same reasons as in the methylation and acetylation studies. The FAs were chosen based on their interaction with FABP7 and because they cover a range of carbon chain length, saturation and therefore three-dimensional structure. This is important when looking for alterations in expression because the FA structure can influence the way it interacts with other fats, proteins and even DNA. Such features of each FA are shown in table 1.4

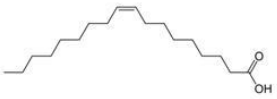
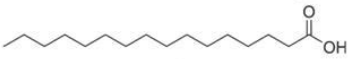
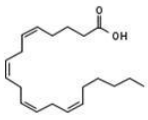
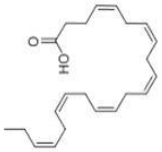
Fatty Acid	Saturation	Carbon chain length	Structure
Oleate	Monounsaturated	18	
Palmitate	Saturated	16	
Arachidonic acid	Polyunsaturated	20	
Docosahexaenoic acid	Polyunsaturated	22	

Table 1.4 Details of the fatty acids used in this work

This table shows the saturation, carbon chain length and schematic structure of each of the fatty acids used in this work.

A dose response experiment was carried out for each of the breast cancer cell lines in order to choose a dose of FA that did not cause extensive cell death; cell death would result in protein degradation and decreases in total protein levels therefore negating any specific effects of the FAs in FABP7 expression.

Palmitate dose ranges of 50μM to 500μM were investigated based on the work by Zeng *et al.*, (2010). MCF-10a, HMT-3522 S1 and MCF-7 breast cancer cell lines were exposed to 100μM, 200μM and 400μM of palmitate resulting in up to 50% cell death. The dose range was extended in this experiment due to the fact that the cell lines were different and would perhaps respond slightly differently. These doses were further confirmed as appropriate based on the research by Hardy *et al.* (2000); doses of palmitate ranging from 10μM up to 400μM were investigated in MDA-MB-231 cells.

Doses of 50μM to 500μM were also investigated for oleate. Dose ranges of 100μM to 400μM have previously been studied in MCF-7 and MCF-10a (Zeng *et al.*, 2010). These doses of oleate have also been found to increase FABP1 expression in the FAO rat hepatoma cell line (Meunier-Durmort *et al.*, 1996). The effect on cell growth of oleate has been studied in MDA-MB-231 cells with a dose range of approximately 1.8-8.9μM; however this was over a much longer time period than was being investigated in this study. Again the dose range was extended due to the fact that MDA-MB-231 and BT-20 cells may respond differently to other breast cancer cell lines.

For investigating the effect of AA on the breast cancer cell lines a range of 1-50μM was selected. Again this was deemed suitable based on information in the literature. For example Razanamahefa *et al*., (2000) tested doses ranging between approximately 8μM and 33μM, with T47D breast cancer cells exposed to AA for up to 3 days. Smaller doses were investigated by Najid *et al*., (1989) (10pM-10μM) in MCF-7 cells but over a time period of up to 10 days. Chamras *et al.*, (2002) also looked at the effect of AA on MCF-7 cells with doses of 1μM-100μM, however again the time points differed from the present study. The BRIN-BD11 rat islet cells were treated with

50, 100 or 150µM of AA for 24 hours (Keane *et al.*, 2011). These doses were found to alter the expression of various genes related to insulin secretion (Keane *et al.*, 2011). Despite the cells not being a breast cell line it was useful to know these doses impact on gene expression.

A range of 10-200µM of DHA was tested to observe the percentage cell death. This range was chosen based on the research by Blanckaert (2010); MDA-MB-231 cells were treated with either 20µM or 100µM over 24, 24 and 72 hours; proliferation and apoptosis were studied. The range was extended to account for variability in dosing and assessment methods and for the reason that the BT-20 cell line may have responded differently. This range was further confirmed rational as Ding *et al.*, (2004) investigated DHA at doses between 15µM and 300µM in MDA-MB-231 and MCF-7 cells; however only at a 72 hour time point.

Bonferroni's multiple comparison test was carried out using Graphpad Prism 5 to determine the most appropriate dose of each FA to use to investigate the expression of FABP7 in the breast cancer cell lines MDA-MB-231 and BT-20 cell lines. The data in figures 1.16 & 1.17 show the percentage cell death and total cell numbers, dead and alive, for MDA-MB-231 and BT-20 cells treated with each FA for 24 hours.

Figure 1.16 Fatty acid dose responses in MDA-MB-231 cells

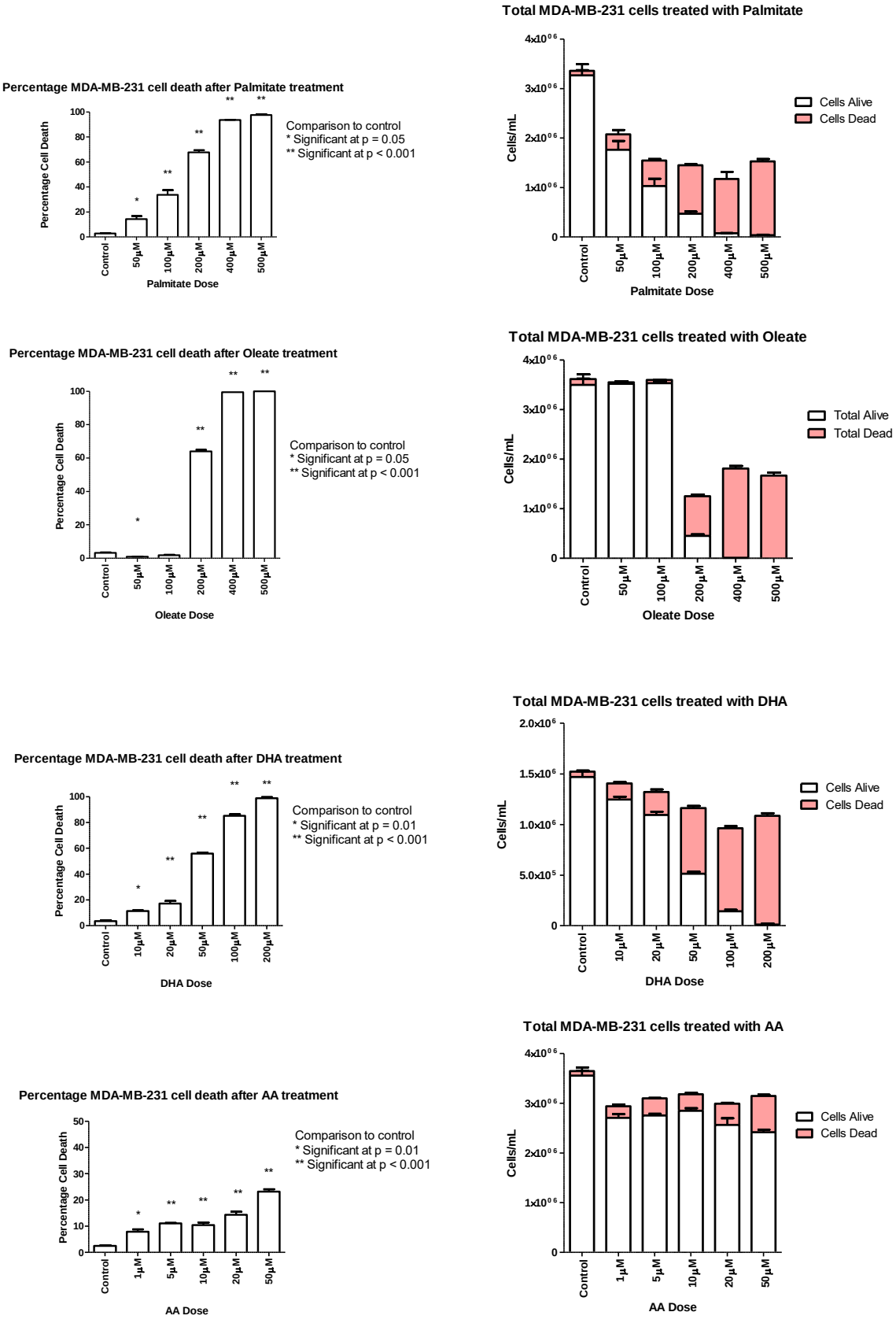
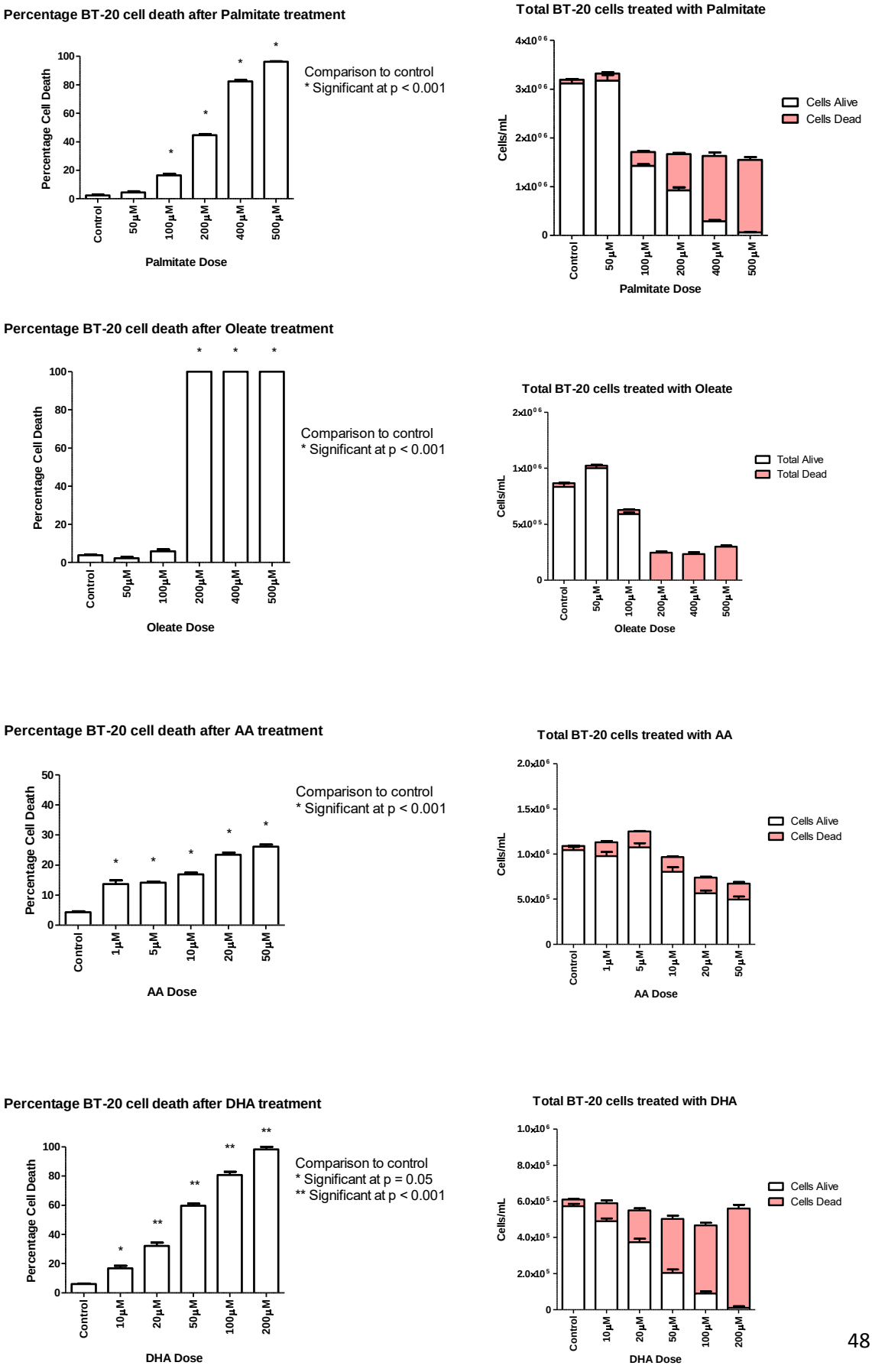


Figure 1.17 Fatty acid doses responses in BT-20 cells



For palmitate a final dose of 50µM was chosen for both cell lines. In the MDA-MB-231 cells there was a significant difference in cell death compared to the control at this dose ($p=0.05$) and it may also be growth inhibitory as there were less total cells compared to the control. However there were still a mean of over 85% of cells alive and the actual number of cells alive was considered enough to give a good RNA yield; therefore 50µM was deemed a high enough dose to exert an effect without causing an undesirable amount of cell death or growth inhibition. A dose 50µM of palmitate was also chosen for BT-20 cells as there was no significant difference in cell death and no reduction in total cell numbers compared to the control. A dose of 100µM palmitate was not chosen for BT-20 cells, even though it gave similar average percentage cell death (16.5%) and apparent growth inhibition as 50µM of palmitate in MDA-MB-231 cells, as it was considered preferential to have as little cell death as possible with a dose high enough to exert an effect.

A dose of 100µM oleate was selected for both cell lines because there was no significant increase in percentage cell death compared to the control and the total cell numbers indicated little growth inhibition. Interestingly 50µM oleate significantly decreased cell death ($p=0.05$) compared to the control in MDA-MB-231 cells; there was a trend for 100µM oleate to also decrease cell death although this was not statistically significant. There was also a trend for 50µM oleate to protect against cell death in the BT-20 cells again this was not statistically significant. A dose of 200µM of oleate in both cell lines considerably increased cell death to a mean of 64.1% ($p<0.001$) and 100% ($p<0.001$) in MDA-MB-231 and BT-20 cells respectively.

For AA treatments 10µM was chosen as a suitable dose for both MDA-MB-231 cells and BT-20 cells. In the MDA-MB-231 cell lines this dose caused 10.5% cell death and may have been slightly growth inhibitory judging by the total cell numbers. In the BT-20 cells 10µM of AA caused a mean

cell death of 16.9% and again may have been slightly growth inhibitory compared to the total cell numbers of the control. Increasing the dose to 20μM increased mean cell death to 14.4% in the MDA-MB-231 cells however this was not statistically significant; in the BT-20 cells the increased dose significantly increased mean cell death to 23.4% ($p<0.001$). A lower dose was not chosen because there was no significant difference in mean cell death between the 1μM, 5μM and 10μM AA doses.

DHA dosing was regarded as appropriate at 10μM in both cell lines. There was little if any growth inhibition as the total cell numbers were comparable to the controls in both cell lines. The percentage cell death was significant compared to the control at a dose of 10μM ($p=0.05$); however at 20μM there was further increased mean cell death in particular in the BT-20 cell line from 16.8% to 32.1% ($p<0.001$).

1.10.3 FABP7 over-expression studies

After investigating what regulates FABP7 with AZA, TSA and FA treatments, the effect of FABP7 over-expression on breast cancer cells was studied. BT-20 cells were chosen to transiently over-express FABP7 because they are of TN and basal-like phenotype. Following transfection with either a control or FABP7 vector, BT-20 cells were counted to study proliferation and cell death. The cells were also treated with FAs and an apoptosis inducer staurosporine, to investigate whether FABP7 protected against cell death. PCR was used to look for changes in expression of genes involved in apoptosis.

1.10.4 Patient demographics

To investigate the expression of FABP7, IGFBP-2 and PTEN in patient samples a Malaysian TN breast cancer cohort was used. Tissue samples from breast cancer patients reported as being

negative for ER, PR and Her2 and diagnosed between 2004 and 2009 at the University of Malaya Medical Centre, were selected for this study. TN phenotype was confirmed by using immunohistochemistry for ER PR and Her2 (section 2.1) and positive cases excluded from further analysis. The age of onset of breast cancer in the patients ranged from 23 to 83 years old with a median of 53 years. The majority of cases (93%) were invasive ductal carcinomas, with the remaining being either medullary or metaplastic cancers.

1.11 Overall Aims

To study the expression of the metabolic biomarkers FABP7, IGFBP-2 and PTEN in clinical cases of Malaysian TN breast cancer. To use appropriate cell lines in order to more fully understand whether epigenetic mechanisms and FAs regulate FABP7 expression. To over-express FABP7 in a breast cancer cell line to further understand the role of FABP7 in breast cancer.

1.12 Overall Hypotheses

The expression of the metabolic biomarker FABP7 is associated with patient survival and clinicopathological parameters in TN breast cancer. FABP7 in breast cancer cell lines is regulated by epigenetic mechanisms and FAs. Over-expression of FABP7 in cell lines alters cell survival and growth. The metabolic biomarkers IGFBP-2 and PTEN are inversely associated in patient samples and are also associated with clinicopathological parameters in TN breast cancer

2 General Materials and Methods

2.1 Immunohistochemistry

All reagents used were from Fisher Scientific (Loughborough, UK) unless otherwise stated.

2.1.1 Tissue section details

One hundred and twenty-two triple negative breast cancer cases were selected for use in this study from the University of Malaya with full ethical approval. The tissue had been formalin fixed and paraffin processed. Tissue sections 3µm thick were cut using a Leica microtome and then floated onto a water bath and picked up onto Tissue-Tek® adhesive slides (ThermoFisher Scientific Surrey, UK). Between 10-20 tissue sections were cut for each case. To confirm TN phenotype all of the cases were IHC stained for ER, PR and Her2; any cases that were positive for the markers were excluded from the study.

2.1.2 Buffers

Sodium citrate buffer (10mM sodium citrate) for antigen retrieval was made by dissolving 29.4g of sodium citrate in 10 litres of ddH_2O and adding 54mL of HCl. The pH was adjusted to pH 6.0 by adding a few drops of 1M HCl. After thorough mixing and the desired pH reached the buffer was stored at 4^oC (Bancroft & Gamble, 2002). Tris-EDTA buffer (10mM Tris, 1mM EDTA) for antigen retrieval was made by dissolving 12g of Tris hydroxymethylamine (Tris and 3.7g of ethylenediaminetetraacetic acid (EDTA, di-sodium salt) in 10 litres of ddH_2O. After mixing thoroughly the pH was adjusted to pH 9.0 by adding a few drops of 1M HCl. The buffer was stored at 4^oC. Tris buffered saline (TBS) wash buffer (0.5M Tris) was made by dissolving 88g of sodium chloride and 6.05g of Tris in 10 litres of ddH_2O and adding 44mL of HCl. The pH was

adjusted to pH 7.4 by adding a few drops of 1M HCl. After thorough mixing and the desired pH reached the buffer was stored at 4^{o}C (Bancroft & Gamble, 2002).

2.1.3 Antigen Retrieval

Twenty of the tissue sections at a time were loaded into a plastic rack and prepared for antigen retrieval by deparaffinising in two baths of Histoclear for five minutes each. The tissue sections were then rehydrated by being taken through two baths of 100% industrial methylated spirits (IMS) for five minutes each and then left in a bath of gently running tap water for a few minutes. The slides were then treated with 0.3% hydrogen peroxide (H_2O_2) (30% H_2O_2 diluted 1:100 with d H_2O) for five minutes to remove any endogenous peroxidase activity and washed thoroughly in dH_2O before being placed in a plastic container with 750mL of retrieval buffer (Rhodes *et al.*, 2010). A lid was put on the container and then the container placed in a Panasonic NE-664 650W microwave for heating to 100^{o}C for 30 minutes on the high setting (Rhodes *et al.*, 2010). After 30 minutes the slides were carefully removed and cooled by gently running tap water in to the container. After this stage it was very important not to let the slides dry out at any time as this would have resulted in weak, poor quality staining. The slides were then placed on a staining tray and rinsed thoroughly with TBS so they were ready to be stained. If less than 20 slides were being used the rack was filled up with blank slides to ensure even and reproducible heat distribution.

2.1.4 Immunohistochemistry Kits

The Universal Elite Kit (Vector Laboratories, Peterborough, UK) comprised of blocking serum-(Normal Horse Serum), secondary antibody, (Biotinylated Universal antibody anti-rabbit/mouse IgG, Heavy & Light chain) and Vectastain Elite ABC reagent. The goat IgG Elite kit (Vector Laboratories, Peterborough, UK) comprised of blocking serum, (Normal Rabbit Serum) secondary antibody, (Biotinylated Anti-Goat IgG, Heavy & Light chain) and Vectastain Elite ABC reagent. The

Elite ABC kits were chosen because of their increased sensitivity so that smaller amounts of protein could be detected and so that a higher dilution of primary antibody could be used and thus preventing background staining. Diaminobenzidine tetrahydrochloride (DAB) substrate-chromogen system from Dakocytomation (Cambridge, UK) was used for visualisation and made according to the instructions and prepared fresh just before use.

2.1.5 Details of primary antibody clones

The details of the primary antibody clones can be seen in table 2.1.

Antibody Clone	Antigen	Type of Antibody	Supplier	Antigen Retrieval Buffer	Elite Kit	Dilution
6F11	ER-α	Monoclonal Mouse	Novacastra Labs (Newcastle-upon Tyne, UK)	Citrate pH 6.0	Universal	1:100
SP2	PR (A & B)	Monoclonal Rabbit	Lab Vision Ltd (Runcorn, UK)	Citrate pH 6.0	Universal	1:200
SP3	HER2	Monoclonal Rabbit	Lab Vision Ltd (Runcorn, UK)	Citrate pH 6.0	Universal	1:400
D5/16 B4	CK5/6	Monoclonal Mouse	Dakocytomation (Cambridge, UK)	Tris-EDTA pH 9.0	Universal	1:300
LL002	CK14	Monoclonal Mouse	Novacastra Labs (Newcastle-upon Tyne, UK)	Citrate pH 6.0	Universal	1:150
6H2.1	PTEN	Monoclonal Mouse	Dakocytomation (Cambridge, UK)	Tris-EDTA pH 9.0	Universal	1:600
C18	IGFBP-2	Polyclonal Goat	Santa Cruz Biotechnology (Middlesex, UK)	Citrate pH 6.0	Goat IgG	1:700
	FABP7	Polyclonal Goat	R&D systems (Abingdon, UK)	Citrate pH 6.0	Goat IgG	1:400
G13	FABP7	Polyclonal Goat	Santa Cruz Biotechnology (Middlesex, UK)	Citrate pH 6.0	Goat IgG	1:200
C20G5	FAS	Monoclonal Rabbit	New England Biolabs (Hertfordhire, UK)	Citrate pH 6.0	Universal	1:100

Table 2.1 Details of primary antibodies including optimised conditions

This Table shows the details of the primary antibodies used in immunohistochemistry experiments. It also shows the final optimised dilution, antigen retrieval buffer and type of Elite kit used in combination with the particular primary antibody.

2.1.6 Controls

As whole tissue sections were used rather than tissue microarrays; where possible, normal components within the tissue section were used as an internal control. Omission of the primary antibody controls were also carried out to ensure the specificity of the secondary antibody; a selection of cases that had previously stained positive for each antibody were incubated overnight in TBS rather than primary antibody.

2.1.6.1 Oestrogen receptor, Progesterone receptor and Her2 receptor controls.

Formalin fixed, paraffin processed cell lines with known expression of each of the receptors were used as positive controls as described previously (Rhodes *et al.*, 2010). Additionally, where possible, normal ducts within the tissue section were used as internal controls.

2.1.6.2 IGFBP-2 antibody control

To test the specificity of the anti-IGFBP-2 antibody an IGFBP-2 blocking peptide (Santa Cruz Biotechnology, UK) was used. The blocking peptide was used at five times the concentration of the antibody (Santa Cruz Biotechnology, technical support); for example as the concentration of the antibody and blocking peptide were the same, 5µL of blocking peptide, 1µL of antibody in 500µL of TBS. The antibody and blocking peptide were incubated together in a tube for two hours at room temperature. Following this, 194µL of TBS was added to achieve the correct dilution of primary antibody. This solution was then used for incubation overnight at 4^{o}C instead; this was used on a selection of cases that had previously stained positive using the anti-IGFBP-2 antibody.

2.1.6.3 FABP7 antibody control

Two FABP7 antibodies were tested, the R&D systems (Abingdon, UK) antibody was chosen as it gave less background staining than the Santa Cruz Biotechnology (UK) antibody. However as both

antibodies resulted in very similar staining patterns the Santa Cruz Biotechnology (UK) antibody was used to verify the results of the R&D systems (Abingdon, UK) antibody staining as a FABP7 blocking peptide (Santa Cruz Biotechnology, UK) was available. This was used as follows; the FABP7 blocking peptide was used at five times the concentration of the antibody (Santa Cruz Biotechnology, technical support); for example 5µL of blocking peptide, 1µL of antibody in 200µL of TBS. The antibody and blocking peptide were incubated together in a tube for two hours at room temperature. Following this, 194µL of TBS was added to achieve the correct dilution of primary antibody. This solution was then used for overnight incubation at 4^{o}C on a selection of cases that had previously stained positive using both FABP7 antibodies. FABP7 staining of normal breast ducts and adipose tissue served as a useful internal positive control (Alshareeda *et al.*, 2012).

2.1.7 Haematoxylin counterstain

Haematoxylin was prepared according to Bancroft & Gamble (2002). Haematoxylin (2.5g) was dissolved in 25mL of 100% ethanol and then added to 500mL of warm distilled water with 50g of potassium alum added. The mixture was then bought to the boil quickly and 0.5g of sodium iodate was added slowly. The flask was chilled in an ice bath and once cool 20mL of glacial acetic acid was added. The stain was filtered before use.

2.1.8 Immunohistochemical staining method

Following antigen retrieval the slides were washed in TBS and then incubated in the blocking serum for 20 minutes. Next the sections were incubated with the primary antibody diluted in TBS, overnight at 4^{o}C; this incubation was used as it gives lower background staining. After a thorough wash with TBS, incubation with the secondary antibody took place for 30 minutes. The slides

were briefly rinsed with TBS and then incubated with ABC reagent for 30 minutes. The sections were washed in TBS and then distilled water and then DAB solution was left on them for 10 minutes. The slides were washed thoroughly in tap water and then counterstained in Harris's Haematoxylin for two minutes. The sections were differentiated in 1% acid alcohol (1% HCl in 70% ethanol) for a few seconds, and then immersed in tap water to allow a colour change for purple to blue in alkali conditions (blued in tap water). They were then dehydrated, cleared and mounted in distyrene plasticizer and xylene mountant (DPX).

2.1.9 Evaluation of immunohistochemistry results

Interpretation of the staining for the markers was carried out by 2 individuals to ensure reproducibility of the results. The basal-like phenotype was determined as cytoplasmic positivity of CK5/6 and/or CK14 in over 10% of the tumour with appropriate staining in normal glands as a positive internal control (Zhang *et al.*, 2010).

The Allred scoring system was used to assess nuclear ER and PR staining, as outlined in table 2.2. Staining of Her2 was assessed with the scoring system in table 2.3 as used in a clinical setting (Wolff *et al.*, 2007). A modified Allred scoring system was used to score the staining for IGFBP-2 and FABP7 as shown below in table 2.2; except it was cytoplasmic staining rather than nuclear staining that was assessed. Cytoplasmic staining was scored on intensity (score 0-3) and proportion (score 0-5); these two scores were added together to given a final score between 2 and 8 (table 2.2). For the purposes of comparing IGFBP-2 expression, with PTEN loss and other clinicopathological variables, a cut-off point score of >5 was used to define IGFBP-2 positivity. This cut off was chosen based on the results from So *et al*. (2008); where intermediate and strong staining of IGFBP2 (which equates to an Allred score of >5), was associated with worse breast cancer specific survival in hormone receptor negative disease

HER2 score	Result of IHC stain
0	No staining
1+	Weak or incomplete membrane staining in any amount of tumour cells
2+	Complete membrane staining that is either weak or non-uniform in >10% of tumour cells
3+	>30% of tumour cells have intense uniform staining

Table 2.2 The Allred scoring system applied to ER, PR, IGFBP-2, FABP7 and FAS staining.
The proportion score and intensity score were added together to reach an overall score. In the case of IGFBP-2 a cut off point of >5 was used to define positivity; so as a minimum positive cases would have strong staining in 1-10% of cells or weak staining in 33-66% of cells.

Proportion score	Proportion of Positively staining cells	Intensity Score	Observation of intensity
0	none	0	Negative staining
1	Up to 1%	1	Weak Staining
2	1%-10%	2	Intermediate Staining
3	10%-33%	3	Strong Staining
4	33%-66%		
5	66%-100%		

Table 2.3 Her2 IHC scoring system

Incomplete membrane staining is defined as positive staining in areas of the cell membrane i.e. the whole cell membrane is not stained positive. Complete membrane staining is defined by the whole cell membrane staining positive. Please see figure 1.5 for a visual representation of this.

Assessment of PTEN loss; the majority of tumours were of large size and heterogeneous in nature, with respect to PTEN expression. Consequently, PTEN expression was considered lost if there was complete absence of staining (both cytoplasmic and nuclear) in at least two thirds of the invasive tumour compartment, with PTEN staining in the adjacent normal stromal tissue being used as the internal positive control.

2.2 Cell Culture

2.2.1 Cell Line Details

The immortalised breast cancer cell lines BT-20, MBA-MB-453, MBA-MB-361, MDA-MB-231 and the non-tumourigenic breast cell line MCF10A were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). The immortalised glioma cell line U251 was obtained from the Health Protection Agency (Salisbury, UK).

2.2.2 Cell Culture Conditions

All cell culture media components were purchased from Lonza (Slough, UK), unless otherwise specified. Cells were grown according to supplier's instructions. Cells were grown in Greiner Bio-one (Gloucester, UK) filter lid tissue culture flasks or tissue culture plates (size stated as appropriate). Phosphate-buffered saline (PBS) was made by dissolving PBS tablets (Fisher Scientific, Loughborough, UK) in ddH_2O and then autoclaving. Costar serological pipettes and tissue culture plates were purchased from Fisher Scientific (Loughborough, UK). A Hettich Mikro200 centrifuge was used for pelleting cells.

2.2.3 BT-20 cell line culture

Eagle's Minimum Essential Medium (EMEM) supplemented with 10% Foetal Bovine Serum (FBS), and 2mM L-glutamine (LG) was used to grow the cells. The cells were cultured in cell culture flasks at 37^{o}C in 5% carbon dioxide in a humidified atmosphere. BT-20 cells are adherent and so upon reaching 80-90% confluence they were washed in cell dissociation buffer (CDB) (Invitrogen; Paisley, UK) and then incubated with fresh CDB for 10 minutes at 37^{o}C. To remove the cells from the bottom of the flasks they were incubated for approximately 10 minutes at 37^{o}C in a mixture of CDB 0.25% Trypsin/0.53mM EDTA (Sigma-Aldrich, Dorset, UK); however they were observed

carefully at intervals to ensure they were not overexposed. To inactivate the trypsin, growth medium was added to the flask; the cell suspension was then transferred to a tube and placed in a centrifuge for three minutes at 16100g. The supernatant was removed and the cell pellet resuspended in 10mL of medium and the cells then passed through a needle 10 times using a syringe, to separate them. An aliquot was removed for counting and then the cells were re-seeded at a density of 0.5 x 10^6 cells/T75 flask.

2.2.4 MDA-MB-361 cell line culture

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FBS and 2mM LG was used to grow the cells. The cells were cultured in cell culture flasks at 37^{o}C in 5% carbon dioxide in a humidified atmosphere. MDA-MB-361 cells are adherent and on observation tended to grow in mounds rather than spread out across the bottom of the flask. Upon reaching what was determined as an appropriate confluency, i.e. when the mounds were large and quite close together covering over 60% of the bottom of the flask, the cells were washed twice in sterile phosphate-buffered-saline (PBS). They were then incubated with 0.25% Trypsin/0.53mM EDTA (Sigma-Aldrich, Dorset, UK) for approximately 10 minutes at 37^{o}C until the cells became detached from the flask. The cells were then treated as described above ready for counting or reseeding; MDA-MB-361 cells were seeded at a density of 0.9 x 10^6 cells/T75 flask.

2.2.5 MDA-MB-453 and MDA-MB-231 cell line culture

DMEM supplemented with 10% FBS and 2mM LG was used to grow the cells. The cells were cultured in cell culture flasks at 37^{o}C in 5% carbon dioxide in a humidified atmosphere. MDA-MB-453 and MDA-MB-231 cells are adherent and so upon reaching 80-90% confluence they were washed twice in sterile PBS. To remove the cells from the bottom of the flasks they were incubated for approximately 10 minutes at 37^{o}C in 0.25% Trypsin/0.53mM EDTA (Sigma-Aldrich,

Dorset, UK), until the cells became detached from the flask. The cells were then treated as previously described ready for counting or reseeding; MDA-MB-453 cells and MDA-MB-231 cells were seeded at a density of 0.5 x 10^6 cells/T75 flask.

2.2.6 MCF10A cell line culture

DMEM:F12 (Invitrogen; Paisley, UK) was supplemented with 5% heat inactivated horse serum, 2mM LG, and 1% hydrocortisone. Additionally the medium was supplemented with 143IU of Insulin Actrapid (Novo Nordish, Bagsvaerd, Denmark), 0.01% Cholera toxin (List Biologicals, Surrey UK) and 0.01% Epidermal Growth Factor. The cells were cultured with the medium in cell culture flasks at 37^{o}C in 5% carbon dioxide in a humidified atmosphere. MCF10A cells are adherent and so upon reaching 80-90% confluence they were washed in cell dissociation buffer (CDB) (Invitrogen; Paisley, UK) and then incubated with fresh CDB for 10 minutes at 37^{o}C. To remove the cells from the bottom of the flasks they were incubated for approximately 10 minutes at 37^{o}C in a mixture of CDB and 0.25% Trypsin/0.53mM EDTA (Sigma-Aldrich, Dorset, UK); however they were observed carefully at intervals to ensure they were not overexposed. The cells were then treated as described previously ready for counting or reseeding; MCF10A cells were seeded at a density of 0.5 x 10^6 cells/T75 flask.

2.2.7 U251 cell line culture

EMEM supplemented with 10% FBS, 2mM LG, 1% Non Essential Amino Acids (NEAA) and 1% Sodium Pyruvate (NaP) was used to grow the cells. The cells were cultured in cell culture flasks at 37^{o}C in 5% carbon dioxide in a humidified atmosphere. U251 cells are adherent and so upon reaching 70-80% confluence they were washed in PBS. To remove the cells from the bottom of the flasks they were incubated for approximately 10 minutes at 37^{o}C in 0.25% Trypsin/0.53mM

EDTA (Sigma-Aldrich, Dorset, UK). However they were observed carefully at intervals to ensure they were not overexposed. To inactivate the trypsin, growth medium was added to the flask; the cell suspension was then transferred to a tube and placed in a centrifuge for three minutes at 16100 g. The supernatant was removed and the cell pellet resuspended in 10mL of medium and the cells then passed through a needle 10 times using a syringe, to separate them. An aliquot was removed for counting and then the cells were re-seeded at a density of 0.5 x 10^6 cells/T75 flask.

2.3 Cell Treatments

2.3.1 Growth Media and controls for Cell Line Treatments

To make serum free medium (SFM) for use in cell line treatment experiments, DMEM:F12 (Invitrogen; Paisley, UK) was supplemented with 0.02% bovine Serum albumin (BSA) (Sigma-Aldrich Dorset, UK) 0.12% Sodium Bicarbonate and 2mM LG. For experiments using fatty acids SFM was made omitting the BSA. SFM media was used for cell line treatment experiments so that FBS did not negate any effects of the specific treatments.

Control vessels of the cell lines were set up alongside the cells to be treated but instead of being subjected to the treatment the cells in these vessels remained in serum free medium unless otherwise stated.

2.3.2 Trichostatin A

Trichostatin A (TSA) was purchased from Tocris Bioscience (Bristol, UK) for acetylation of FABP7 studies; 500µL of DMSO was added to create a 6614.4µM stock solution. The stock solution was aliquoted and stored at -20^{o}C until use. A 500µM working solution was prepared by taking 5µL of 6614.4µM stock and adding it to 2992.5µL of SFM. BT-20 and MDA-MB-231 cells were seeded into T25 flasks at 0.5 x 10^6 cells/ flask, in their respective growth media. Growth medium was replaced 24 hours later with 3mL of SFM. Following 24 hours in SFM the cells were dosed with

either 0.25µM or 0.5µM TSA by adding 7.5µL or 15µL of 500µM working solution respectively to the flasks. These doses were chosen based on dose response curves generated from previous work by (pre-published Zeng *et al*, University of Bristol) and because similar doses have been used to demonstrate the re-expression of the ER by TSA in breast cancer cell lines (Yang *et al.*, 2000). The cells were exposed to TSA for 24 hours and were then subjected to either RNA or protein extraction.

2.3.3 5-Aza-2'-deoxycytidine

5-Aza-2'-deoxycytidine (AZA) (Sigma-Aldrich, Dorset, UK) stock solution, for methylation of FABP7 studies, was prepared by dissolving 1mg of AZA in 1mL of dH_2O to give a 4382µM solution. This solution was then passed through a 0.2µM filter to sterilise it. A 500µM stock was then prepared using the 4382µM solution and SFM. This two-step dilution allowed for appropriate volumes and doses to be used to treat the cells. BT-20 and MDA-MB-231 cells were seeded into T25 flasks at 0.5 x 10^6 cells/ flask, in their respective growth media. Growth medium was replaced 24 hours later with 3mL of SFM. Following 24 hours in SFM the cells were treated with 1µM or 2.5 µM AZA. After 24, 48 or 72 hours of exposure the cells were subjected to either protein or RNA extraction. These doses of AZA and time points were used as they have been used to demonstrate the re-expression of the ER in ER negative breast cancer cell lines (Ferguson *et al.*, 1995).

2.3.4 Fatty Acid dose responses in cell lines BT-20 and MDA-MB-231

For each FA a dose response was carried out to ensure the appropriateness of each dose. Cells were seeded into 6 well plates at a density of 0.15X10^6/well. The following day the medium was changed to SFM. After 24 hours in SFM the cells were dosed with 1.5mL of medium containing the FA; FA doses were made up as in appendix I. Serum free growth medium was supplemented with Fatty acid free BSA (Sigma-Aldrich, Dorset, UK) so the final concentration used was 0.1%.

This ensured that any effect observed would be from the FA of interest rather than from FAs in BSA. The BSA also bound the FA to aid solubilisation and to make the FA available to the cells. The various concentrations of fatty acid free BSA as in appendix I were made up in BSA free SFM and then sterile filtered. The final concentration of ethanol was considered negligible at <0.1%. The control well received SFM with 0.1% fatty acid free BSA. The cells were exposed to the FA for 24 hours and were then counted, as described below, after staining with Trypan blue solution (Fisher Scientific, Loughborough, UK). The following methods for solubilising the FAs palmitate and oleate has been previously carried out by collaborators (Zeng *et al.*, 2010), this method was also found suitable for AA and DHA.

2.3.5 Palmitate

A 400mM stock solution of palmitate was prepared by adding 103mg of palmitate to 1mL of sterile filtered ethanol and heating until the palmitate was fully dissolved. The doses tested were 50µM, 100µM, 200µM, 400µM and 500µM. Solutions were heated and thoroughly mixed at every stage to ensure the palmitate was in solution. This range of doses was chosen based on research findings using palmitate to treat cultured cells (appendix I).

2.3.6 Oleate

A 400mM stock solution of oleate was prepared by adding 38.1µL of oleate solution to 261.9µL of sterile filtered ethanol. The solution was heated and mixed thoroughly. The doses tested were 50µM, 100µM, 200µM, 400µM and 500µM. Solutions were heated and thoroughly mixed at every stage to ensure the oleate was in solution. This range of doses was chosen based on research findings using oleate to treat cultured cells (appendix I).

2.3.7 Arachidonic acid

A 40mM stock solution of arachidonic acid (AA) was made by adding 3.84µL of AA to 296.16µL of sterile filtered ethanol. The solution was heated and mixed thoroughly. The doses tested were 1µM, 5µM, 10µM, 20µM and 50µM. Solutions were heated and thoroughly mixed at every stage to ensure the AA was in solution. This range of doses was chosen based on research findings using AA to treat cultured cells (appendix I).

2.3.8 Docosahexaenoic acid

A 40mM stock solution of docosahexaenoic (DHA) acid was made by adding 2.79µL of DHA to 297.21µL of sterile filtered ethanol. The solution was heated and mixed thoroughly. The doses tested were 10µM, 20µM, 50µM, 100µM and 200µM. Solutions were heated and thoroughly mixed at every stage to ensure the DHA was in solution. This range of doses was chosen based on research findings using DHA to treat cultured cells (appendix I).

Final conditions used for each FA are stated in the appropriate chapters.

2.3.9 Cell counting using Trypan blue

Cell counting using Trypan blue was used to ensure cells were seeded at a correct and consistent density and to assess the impact on cell death of treatments used. Trypan blue counting has been found to give results consistent with other methods to assess cell viability such as flow cytometry (Gill *et al.,* 1997) and PARP cleavage by western blotting (Thomas *et al*., 2010). Cells were removed from the culture vessel using trypsin and then pelleted as previously described. Cells were then fully re-suspended in a known volume of growth medium to allow calculation of total cells per culture vessel. Cell suspension (30µL) was mixed with 30µL of Trypan blue solution and then added to a chamber of a Neubauer haemocytometer. Live and dead cells were counted separately in both chambers of the haemocytometer to take into account the dilution factor of

the Trypan blue; live cells were bright whilst dead cells were dark blue upon observation down the microscope. Total cells, alive and dead, were then calculated using the following equation:

Total number of cells (dead and/or alive) = (cells counted x 10^4) x volume of cell suspension in mL

2.4 Protein extraction

2.4.1 Lysis Buffer

Lysis buffer was made using the components in table 2.4; they were from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

	To make 1L of lysis buffer	
	Quanitity (g)	Final Concentration
Tris HCL	1.576	10mM
Sodium Chloride	2.92	50mM
EDTA	1.86	5mM
Sodium Pyrophosphate	6.66	15mM
Sodium Fluoride	2.1	50mM
Sodium Orthovanadate	10mls of 100x solution	100µM

Table 2.4 Lysis buffer composition

To make the 100x sodium orthovanadate solution 36.8mg of sodium orthovanadate was dissolved in 20mL dH_20. The solution was adjusted to pH10 so it turned yellow. It was then boiled until it became clear. The substances above in the table were then added to dH_2O to make up 1L of solution. The lysis buffer was stored at 4^oC; when required for use 10µL each of protease inhibitor cocktail and phosphatase inhibitor cocktail 2 was added to every 1mL of lysis buffer to inhibit protein degradation and loss of phospho groups. Both cocktails are commercially available from Sigma-Aldrich (Dorset, UK)

2.4.2 Cell Lysate Preparation

Cells were either lysed directly in the culture vessels after washing twice with PBS or removed from the bottom of the culture vessel by trypsinisation and pelleted by centrifuging at 16100g for three minutes. Depending on the size and number of cells in the pellet or culture vessel, between 20-500µL of lysis buffer, with 10µL phosphatase and protease inhibitor cocktails per 1mL of lysis buffer (Sigma-Aldrich, Dorset, UK), was added and gently mixed. This was incubated for 30 minutes at 4^oC with gentle agitation. The lysate was then collected, clarified by centrifugation for

15 minutes at 4^{o}C (18900g) and the supernatant collected. The resulting pellet was discarded and the lysate supernatant was then stored at -20^{o}C until required for use.

2.4.3 Protein quantification of cell lysates

The Pierce BCA protein assay kit (Fisher Scientific Loughborough, UK) was used according to the kit instructions. The kit comprised of two solutions that were mixed just before use in a 1:50 ratio to make the working solution. Briefly 5µL of protein standards of known concentration ranging between 5-250µg/µL and 5µL of samples were incubated in individual wells of a 96 well plate with 200µL of working solution from the kit for 30 minutes. Protein standards and all samples were measured in duplicate. Samples were tested neat and at 1:5 and 1:10 dilutions to ensure a result that would be within the range of the standards. After incubation the plate was put onto a Labsystems Multiskan Plus plate to be read at a wavelength of 540nm. The plate reader was connected to a computer with Genesis plate reader software that then read the light absorbance of the samples in the wells and calculated the concentration using a standard curve generated from the protein standards.

The pierce 660nm protein assay kit (Fisher Scientific Loughborough, UK) was used according to manufacturer's instructions for samples lysed in x2 Laemmli buffer. Briefly 10µL of protein standards of known concentration ranging between 5-250µg/µL and 10µL of samples were incubated in individual wells of a 96 well plate with 150µL of working solution from the kit for 5 minutes. Samples were diluted and analyzed as above except read at a wavelength of 660nm.

2.5 Western Blotting

All reagents were purchased from Fisher Scientific (Loughborough, UK) unless otherwise stated. Any modifications to this method are stated in the relevant chapters.

2.5.1 Buffers and Solutions

A 10x running buffer stock was made by dissolving 30.3g of Tris, 144g of glycine and 10g of sodium dodecyl sulphate (SDS) in 1 litre of ddH_2O. When required for use the 10x running buffer was diluted 1:10 in ddH_2O to the volume required to make 1x running buffer.

A 10x transfer buffer stock was made by dissolving 30.3g of Tris and 144g of glycine in 1 litre of ddH_2O. When required for use the 10x transfer buffer was diluted by taking 100mL of it and adding 200mL of methanol and 700mL ddH_2O to make 1x transfer buffer.

A 10x washing buffer stock (TBS-T 1% Tween 20) was made by dissolving 24.2g of Tris (tris hydroxymethylamine), 80g of sodium chloride and 10mL of Tween 20 in 1L of ddH_2O. When required for use the 10x washing buffer was diluted 1:10 in ddH_2O to the volume required to make 1x washing buffer.

Ammonium persulphate solution (APS)was made by dissolving 25mg of ammonium persulphate in 100mLs of ddH_2O. Tris 1.5M pH8.8 was made by dissolving 27g of Tris in 150mL of ddH_2O and 2mLs of concentrated HCl. Tris 0.5M pH6.8 was made by dissolving 3g of Tris in 50mL ofddH_2O, the correct pH was reached by adding 1M HCl drop-wise. SDS (10%) was made by dissolving 10g of SDS is 100mL of ddH_2O.

2.5.2 Acrylamide gel electrophoresis

Gels were cast using BioRad (Hertfordshire, UK) glass cassettes and casting stands. The resolving gel contained 12% acrylamide (37.1:1 acrylamide:bisacrylamide) in 1.5M pH 8.8 Tris buffer with 0.12% SDS, 0.12% APS and 0.012% TEMED. The stacking portion of the gel served to "condense" the proteins before entering the resolving portion of the gel; it comprised of 4% acrylamide (37.1:1 acrylamide:bisacrylamide) in 0.5M Tris buffer pH6.8 with 0.1% SDS, 0.1% APS and 0.015% TEMED.. The stacking portion was poured on top of the resolving portion after it had set.

Laemmli sample buffer x2 concentration (Sigma-Aldrich, Dorset, UK), comprising of 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.004% bromophenol blue in 0.125M Tris buffer pH6.8, was added to samples that had been aliquoted out so they would each have the same amount of total protein per tube (the amount of protein used in each experiment is stated in the relevant sections). Samples were heated at 100^{o}C for five minutes on a heat block. Novex Sharp pre-stained ladder (Invitrogen; Paisley, UK) was used to ensure protein separation and identify the proteins of interest by size. The gel was loaded and the BioRad tetra tank (Hertfordshire, UK) was filled with running buffer. The gel underwent electrophoresis at 100V until the dye had run out of the stacking portion into the resolving portion; the voltage was then set to 120V and until the dye front reached the end of the gel unless otherwise stated.

2.5.3 FABP7 positive control

The FABP7 recombinant peptide (Abcam, Cambridge, UK) was loaded onto the gel for use as a positive control during optimisation. The peptide was diluted 1:50 with PBS and then 15µL loaded into the well. U251 cell line lysates were used as a positive control at 30µg/well unless otherwise stated.

2.5.4 Protein Transfer

Hybond-P polyvinyl difluoride (PDVF) membrane (Fisher Scientific Loughborough, UK) was activated in methanol for five minutes then wetted in transfer buffer and sandwiched in a cassette with the gel and pre-wetted filter paper and pads. To ensure good contact between the gel and membrane any bubbles were rolled out. The cassette sandwich was placed in a transfer tank filled with transfer buffer and a cool pack. Transfer took place for one hour at 100V.

2.5.5 Primary and secondary antibodies for protein detection.

Following protein transfer the membrane was blocked in the 5% milk powder (Tesco supermarket, UK) in TBS-T for one hour at room temperature. The membrane was then incubated with primary antibody; details of the dilutions and diluents for each primary antibody can be seen in table 2.5. Incubation took place overnight at room temperature. The following morning the membrane was washed in TBS-T three times for 15 minutes each wash; next it was incubated in secondary antibody for one hour at room temperature; secondary antibody details are in table 2.5. When probing for more than one protein on the same membrane, the membrane was cut in an appropriate place according to the sizes of the proteins of interest and the protein ladder position.

Antigen	Primary Antibody Species, Clone & Supplier	Primary Antibody Diluent	Primary Antibody Dilution	Secondary Antibody Clone & Supplier	Secondary Antibody Diluent	Seconday Antibody Dilution
FABP7	Goat, R&D Systems (Abingdon, UK)	5% Milk in TBS-T	1:500	anti-goat, Sigma-Aldrich (Dorset, UK)	5% Milk in TBS-T	1:2000
FABP7	G13,Goat, Santa Cruz Biotechnology (Middlesex, UK)	5% Milk in TBS-T	1:400	anti-goat, Sigma-Aldrich (Dorset, UK)	5% Milk in TBS-T	1:1500
β-actin	AC-40, Mouse, Sigma-Aldrich (Dorset, UK)	5% Milk in TBS-T	1:10,000	anti-mouse, Sigma-Aldrich (Dorset, UK)	5% Milk in TBS-T	1:5000

Table 2.5 Primary and Secondary antibody details
This table shows the details of the primary antibodies, corresponding secondary antibodies and the optimal conditions for protein detecting following western blotting. All secondary antibodies were horseradish peroxidase labelled. All antibodies were diluted in 5% Milk powder in TBS-T.

2.5.6 Membrane Imaging

Following secondary antibody incubation the membrane was washed again in three changes of TBS-T for 15 minutes each wash. A SuperSignal West Femto Chemiluminescent Substrate kit from Fisher Scientific (Loughborough, UK) was used according to the instructions for visualisation, briefly the two reagents in the kit were mixed in equal volumes to give a working solution. The membrane was incubated with the working solution for five minutes. The membrane was then imaged using a ChemiDocIt system (UVP, Cambridge, UK) connected to a computer with VisionWorksLS software.

2.6 RNA extraction

Two methods of RNA extraction were used depending on the experiment. The ISOLATE RNA Mini Kit (Bioline, London UK) was used due to the ease of use in high-throughput conditions such as in the cell line experiments using AZA, TSA and FAs. The TRI-reagent (Sigma-Aldrich, Dorset, UK) was used in experiments when there were less samples to prepare and high yield was required; for instance, in transfection experiments. The particular method used is stated in the relevant chapters.

2.6.1 RNA extraction using a kit

RNA extraction took place using an ISOLATE RNA Mini Kit (Bioline, London UK); it was used according to kit instructions. The kit was used to help remove genomic DNA (gDNA) and to prevent RNA degradation as the solutions contained inhibitors of RNAses. Briefly growth medium was fully aspirated and cells were then incubated with kit lysis buffer for three minutes. A cell scraper was used to homogenise the cells with the lysis buffer. The homogenate was then transferred to a spin column in a collection tube. It then underwent a series of spins and washes according to the instructions so the RNA bound to the spin column without any contaminants. Finally RNase-free water was incubated in the spin column for one minute and then the column was spun to elute the RNA. The RNA was then stored at -80^{o}C until required for use.

2.6.2 RNA extraction using TRI reagent

RNA extraction took place with TRI reagent according to manufacturer's guidelines. TRI reagent prevented RNA degradation and gave high yield of RNA even with samples with low numbers of cells. Growth medium was removed and an appropriate volume of TRI reagent was added depending on the culture vessel size (1mL per 10cm^2 culture dish area). The TRI-reagent was

passed through a pipette several times to homogenize the sample and then left to incubate at room temperature for five minutes. The homogenised sample was then transferred to a 1.5mL micro-centrifuge tube for processing. Next 200µL (per 1mL of TRI reagent) of chloroform was added to the tube, shaken vigorously and then incubated for 15 minutes at room temperature. The tube was then spun for 15 minutes at 4^{o}C, 16100g. The colourless aqueous phase was transferred to a fresh tube and 500µL of isopropanol (per 1mL of TRI reagent) was added; the tube was vortexed for a few seconds and then allowed to incubate for 10 minutes at room temperature. To pellet the RNA the tube was spun at 16100 g at 4^{o}C for 8 minutes; the supernatant was then discarded and 1mL (per 1mL of TRI reagent) of 75% ethanol was added to the pellet to wash it. The tube was then spun for five minutes at 11200g; the ethanol was removed and the RNA pellet air dried. The RNA was dissolved in nuclease free water by vortexing. The RNA was then stored at -80^{o}C until required for use.

DNase I Amplification Grade treatment kit was purchased from Sigma-Aldrich (Dorset, UK) and used to treat the Tri-Reagent extracted RNA samples to remove genomic DNA. For each sample 1µL of DNase buffer and 1U (1µL) of DNase was added to 2µg of RNA in a volume of 8µL. The mixture was incubated at room temperature for 15 minutes. EDTA stop buffer (1µL) was then added and the mixture incubated at 70^{o}C for 10 minutes; following this the mixture was then cooled on ice. The RNA was then re-quantified to ensure the concentrations of the samples were unchanged from the quantity prior to DNase treatment.

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2.6.3 RNA extraction from formalin fixed paraffin embedded tissue

To help determine whether FABP7 protein levels, as determined by IHC, correlated with mRNA expression, RNA was extracted from formalin fixed paraffin embedded (FFPE) tissue; this was done using an RNeasy FFPE kit (Qiagen, Crawley, UK). Three 10µM thick tissue sections were placed in microcentrifuge tubes and deparaffinized by adding 1mL of histoclear. The tubes were then vortexed for 10 seconds and incubated at 56^{o}C for three minutes; then they were centrifuged at 9450g for two minutes to pellet the tissue. The histoclear wash was repeated a further two times. The histoclear supernatant was discarded and to remove any residual histoclear the tissue was washed in 1mL of ethanol by vortexing and then centrifuging at 9500g for two minutes. This was also repeated another two times. As much of the ethanol was removed as possible without disturbing the pellet; the pellet was then air dried until any remaining ethanol had evaporated. Next 240µL of Buffer PKD was added and mixed by vortexing; it was then spun for one minute at 5600g; 10µL of proteinase K was added to the lower clear phase and mixed with pipetting. The kit instructed to incubate at 56^{o}C for 15 minutes, however, a considerably higher RNA yield, with no change in quality, was obtained by incubating overnight at 56^{o}C. The following morning the tubes were incubated at 80^{o}C for 15 minutes; it was important that the tubes were placed on the heating block for incubation only when the temperature had reached 80^{o}C and were not allowed to gradually warm up. The lower clear phase was transferred to a new tube and chilled on ice for three minutes and then centrifuged for 15 minutes at 10200g. The samples were then DNase treated by adding 25µL of DNase Booster Buffer and 10µL of DNase I stock solution and mixed by inversion. The tubes were pulsed in a centrifuge to collect liquid on the sides of the tubes. The tubes were incubated with the DNase treatment at room temperature for 15 minutes. To adjust the binding conditions 500µL of Buffer RBC was added and mixed and then 1.2mL of ethanol added; 700µL of the sample was transferred to an RNeasy MiniElute spin column in a 2mL collection tube. The spin column was centrifuged for 15 seconds at 5600g. The flow through was discarded and then more sample was added to the columns and the steps

repeated until the entire sample had passed though the column. A volume of 500µL of RPE buffer was added to the column and spun for 15 seconds 5600g.; this was repeated except with a spin of two minutes. The column was transferred to a new collection tube and centrifuged at full speed for five minutes; this ensured there was no ethanol carried over during RNA elution. Finally the column was transferred to a new tube and the RNA eluted with 20µL of nuclease free water by incubation for one minute followed by centrifugation at 9500g for one minute. The RNA was then ready for downstream assays.

2.6.4 RNA quantification

The Nanodrop 1000 spectrophotometer (ThermoFisher Scientific Surrey, UK) pedestals were thoroughly cleaned and then distilled water was used to blank the machine. RNA (1.5µL) was loaded onto the pedestal of the Nanodrop. The Nanodrop then gave an absorbance reading which was converted to an amount of RNA within the Nanodrop software V3.7.1 of the connected computer. Each sample was measured in duplicate and the pedestals wiped thoroughly between each sample. The Nanodrop also gave a 260/280 ratio and a 260/230 ratio. The 260/280 ratio was preferably between 1.76 and 2.1; anything below meant there was significant protein contamination and anything above indicated there was RNA degradation. The 260/230 ratio ideally was around 1.8, anything significantly below this indicated that was contamination from substances such as ethanol, phenols, carbohydrates or salts. Once quantified the RNA could be used for downstream assays.

2.6.5 RNA Integrity

The RNA integrity was tested using agarose gel electrophoresis. A 50x stock solution of Tris-acetate-EDTA (TAE) was prepared and comprised of 20M Tris-acetate and 0.05M EDTA solution

pH8.. The 50x TAE buffer was diluted 1:50 with ddH_2O prior to use. A gel was prepared by heating 1% agarose in 1x TAE buffer (0.4M Tris-acetate, 0.001M EDTA) in a microwave until boiling. Ethidium Bromide solution was added and the mixture swirled to give a final concentration of 50µg/mL. It was then poured into a gel tray with combs to form the wells and allowed to set. Each RNA sample (5µL) was added to 15µL of nuclease free H_2O and 5µL of Crystal 5x DNA loading buffer (Bioline, London UK). Each sample was then loaded into the wells of the gel and the gel underwent electrophoresis at 80V for 20 minutes in TAE buffer. The gel was imaged using a MiniBis UV illuminometer (DNR Bio-Imaging Systems, Stretton Scientific, Derbyshire, UK). The RNA integrity was considered acceptable if there were 2 bands on the gel corresponding to the 18S and 28S ribosomal RNAs and if the 28S band was more intense than the 18S band.

2.7 Complementary DNA synthesis

The resulting RNA was then used for downstream synthesis of DNA to analyse expression of mRNA.

2.7.1 Reverse Transcription polymerase chain reaction (PCR)

1µg of RNA was used for conversion to cDNA. The cDNA synthesis was carried out using the GoScript™ Reverse Transcription system (Promega, Southampton, UK). DNase treated RNA (1µg in a volume of 5.5µL) was added to 10µL of nuclease free H_2O and 1µL/0.5µg of Random Primer for each sample. The samples were then heated to 70 ^oC for five minutes and then chilled on ice for at least five minutes. A reverse transcriptase containing mixture was prepared for n+2 samples to allow for pipetting error. The reaction mixture per sample comprised of 4.0µL GoScript™ 5x Reaction Buffer, 4.4mM $MgCl_2$, 0.5mM of each dNTP/2mM of PCR nucleotide mix, 20U recombinant RNasin® ribonuclease inhibitor and 1.0µL of GoScript™ Reverse Transcriptase. This mixture was then added to each sample (9.82µL).

A minus reverse transcriptase control (-RT) was used as a negative control. The RNA underwent the same treatment with the same reagents except the GoScript™ Reverse Transcriptase was replaced with nuclease free water. It was used as a means of determining whether there was any DNA contamination. A no-template (NT) negative control was also used to ensure there was no DNA contamination from other sources; instead of any RNA being used the sample comprised of 10µL of nuclease free water and was treated as reverse transcriptase positive samples. The samples were then incubated as follows in table 2.6 using a PTC-200 DNA engine gradient cycler.

Step	Temperature (°C)	Time (minutes)
Anneal	25	5
Extend	42	60
Inactivate	70	15
Chill	4	hold

Table 2.6 Reverse Transcription conditions
This table shows the temperature and duration conditions for reverse transcription PCR.

2.7.2 Primers

Primers were designed using Primer Express Version 2.0 (Applied Biosystems, UK); details of the primer sets used are in table 2.7 and table 2.8. Primers were ideally required to have a %GC content between 40 and 60% and each primer of a primer set was to have a similar if not identical melting temperature. Primers were also designed to span exon boundaries or to lie either side of an intron; gDNA contains both introns and exons so designing primers in this way ensured that gDNA was not amplified or the gDNA amplified product was much larger. The β–actin primers were designed by Dr Liku Tereza, who kindly shared the primer sequences for this work. The primers for BCL-X, Caspase 9 and survivin splice variants were kind gifts from Dr Rachel Hagen. Primers were ordered from Eurofins MWG operon (London, UK). They were reconstituted according to manufacturer's instructions and then diluted, to give a 5µM working stock, with nuclease free water ready for use and then were aliquoted and stored at -20^{o}C.

Gene	NCBI Accession Number	Splice Variant	Product Size (bp)		Primer Sequence 5' to 3'	Tm (Melting Temperature ^{o}C)
Survivin	NM_001168	Full Length	450	Forward	GCTCCGGCCAGAGGCCT	65.5
		EX3	330	Reverse	GCATGGGTGCCCCGACG	65.5
		2B	520			
Caspase 9	NM_001229.3	9A	270	Forward	GCTCTTCCTTTGTTCATC	57.9
		9B	740	Reverse	CATCTGGCTCGGGGTTA	63.7
BCL-X	NM_138578.1	BCL-X_S	160	Forward	CATGGCAGCAGTGAAGC	59.4
		BCL-X_L	350	Reverse	GCATTGTTCCCGTAGAGA	59.8
β-actin	NM_00101.3		70	Forward	CCTGGCACCCAGCACAAT	59
				Reverse	GCCGATCCACACGGAGTACT	60

Table 2.7 Standard PCR primer details

This table shows the details of the primers used in standard PCR experiments. The survivin primer sets generated three different products the names and size (bp) are stated. Caspase 9 and BCL-X primer sets gave two products each. B-actin was used as a reference gene.

Gene	NCBI Accession Number	Product Size (bp)		Primer Sequence 5' to 3'	Tm (Melting Temperature ^{o}C)
FABP7	NM_001446.3	70	Forward	GGCTTTCTGTGCTACCGGA	59
			Reverse	CACGCCTAGAGCCTTCATGT	59
β-actin	NM_00101.3	70	Forward	CCTGGCACCCAGCACAAT	59
			Reverse	GCCGATCCACACGGAGTACT	60
Survivin FL	NM_001168.2	433	Forward	GAGCTGCAGGTTCCTTATC	59
			Reverse	ACAGCATCGAGCCAAGTCAT	64.8
Survivin EX3	NM_001012270.1	240	Forward	GGCGGCATGGGTGCCCCGACGTT	61
			Reverse	TGGTTTCCTTTGCATGGGG	63.2
Survivin 2B	NM_001012271.1	266	Forward	GGCGGCATGGGTGCCCCGACGTT	61
			Reverse	GTGCTGGTATTACAGGCGTAAG	62.4
Caspase 9A	NM_001229.3	184	Forward	AGTGGACATTGGTTCTGGAG	61.6
			Reverse	CTTCTCACAGTCGATGTTGG	61
Caspase 9B	NM_001229.3	127	Forward	TGGTGATGTCGAGCAGAAA	64.1
			Reverse	CTGGTCGAAGGTCCTCAAAC	63.8
ER	NM_000125.3	71	Forward	TCTTGGACAGGAACCAGGGA	60.1
			Reverse	TGATGTAGCCAGCAGCATGT	59.75

Table 2.8 qPCR Primer details
This table details the primer sets used in qPCR experiments. The product sizes for each primer set are stated for validation purposes i.e. to test the primers the qPCR products were subjected to agarose gel electrophoresis.

2.7.3 Standard PCR

To ascertain which cell lines expressed FABP7 mRNA standard PCR was carried out using GoTaq® Hot Start Polymerase system (Promega, Southampton, UK). This was used according to manufacturer's instructions. The reaction mix per sample comprised of 5µL 5x Green GoTaq®Flexi Buffer, 1.5mM $MgCl_2$, 0.48mM of dNTP PCR nucleotide mix, 0.5µM each of forward and reverse gene specific primers, 0.1µL of GoTaq® Hot Start Polymerase and 2.5µL of each sample made up to a final volume of 25µL with nuclease free water. The PCR reaction mixtures were incubated in a PTC-200 DNA engine gradient cycler as in table 2.9.

	Temperature (°C)	Time (mins)	
	95	2	
Denature	95	1	
Anneal	59	1	34 cycles
Extension	72	1	
	72	5	
	4	Hold	

Table 2.9 Standard PCR cycling conditions

This table shows the temperatire and duration conditions for each stage of the standard PCR. Thirty-four cycles of denaturing, annealing and extension took place.

2.7.4 Tris-acetate-EDTA (TAE) Buffer

A 50x stock solution was prepared by dissolving 121g of Tris in 240mL of ddH_2O. Acetic acid (28.6mL)and 50mL of 0.5M EDTA solution pH8 was added. Finally ddH_2O was added to make up a final volume of 500mL. The 50x TAE buffer was diluted 1:50 with ddH_2O prior to use.

2.7.5 Agarose gel electrophoresis

A 2.5% agarose gel was made as described in section 2.6.5 for running the PCR products on. Each sample was loaded into wells (10µL) and the gel underwent electrophoresis at 100V for 30 minutes in TAE buffer. The gel was imaged using a MiniBis UV illuminometer (DNR BioImagaing Systems, Stretton Scientific, Derbyshire, UK).

2.7.6 Semi-Quantitative Real Time PCR

A GoTaq® Real-Time PCR system was used (Promega, Southampton, UK). A master mix was made for n+5 reactions to allow for pipetting error. The master mix per reaction comprised of 8µL of

GoTaq® qPCR Master Mix, 200nM each of the gene specific forward and reverse primers and 0.16µL of CRX reference dye made up to a volume of 14.6µL with nuclease free water.

A standard curve was generated by pooling cDNA from each sample together and then serial diluting it as follows: 1:2, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:160. Neat to 1:40 were used as standards for FABP7 and 1:5 to 1:160 were used for the reference gene β-actin. 14µL of the PCR mix was added to wells of a 96 well PCR plate along with 2µL of standard or 2µL of sample, including –RT and NT samples. Standards were plated in duplicate and samples were plated in triplicate. The plate was then spun to ensure all the mix and cDNA was at the bottom of the well and then was placed on a 7300 Applied Biosystems PCR machine connected to 7300 software. Cycling then took place as in table 2.10. In addition, to check the specificity of the primers, dissociation curves were generated by adding the dissociation curve setting to the end of the qPCR cycle (95°C for 15 seconds, 60°C for 1 min, 95°C for 15 seconds and 60°C for 15 seconds), specific primers produced one clean peak, non-specific amplification or primer-dimers were seen as two or more peaks. Additionally the products were run on an agarose gel as described in section 2.7.3 to check product was of expected size and only one product was present for each sample. FABP7 primers were validated against mRNA from U251 cells known to express FABP7. However, U251 mRNA was not included in qPCR experiments as a positive control because it would have prevented any FABP7 signal from the breast cell line samples from being detected.

Temperature (°C)	Time (mins)		
95	10		
Denature 95	15	↓	40
Anneal 60	1	↓	cycles

Table 2.10 qPCR cycling conditions
This table show the cycling conditions for semi-quantitative PCR.

2.7.7 Analysis of qPCR results

Results were analysed using the relative standard curve method. The cycle threshold (Ct) values, generated from the qPCR run, were exported into Microsoft Excel; the Ct values from the set of serially diluted standards were used to plot a standard curve with the equation y=mx+c; acceptable correlation efficiency (R^2 Value) of the data set was set at R^2>0.98. In addition the efficiency was checked by looking at the m value of the standard curve; primers generating m values of 3.2 - 3.6 were determined to be efficient for qPCR (E values = 110%-90%). The Ct values of the samples were then converted using values from the graph equation y=mx+c, as follows: Log Value of Ct was derived from the following equation: Log Value of Ct =((Ct-c)/m) where c and m are from the graph equation generated from the serially diluted standards. These values were then antilogged (10^x). This was done for the Ct Values of both the gene of interest and the chosen reference gene, β-actin. Gene of interest expression values were then normalised to β-actin values so that the relative expression of the gene of interest in each sample could be compared and if required fold change in mRNA calculated.

2.8 Transfection of BT-20 cells

To study the role of FABP7 in TN and BLBC, the breast cancer cell line BT-20 was transiently transfected with a plasmid containing the FABP7 gene.

2.8.1 The pCMV-SPORT6 containing FABP7

The pCMV-SPORT6 vector containing the full length cDNA clone FABP7 (accession number NM_001446.3) was purchased from Thermo-scientific (Surrey, UK); it was in the form of a ready to use glycerol stock of transformed bacteria. A map of the vector can be seen in figure 2.1; the FABP7 gene was inserted between the NotI and SalI restriction sites.

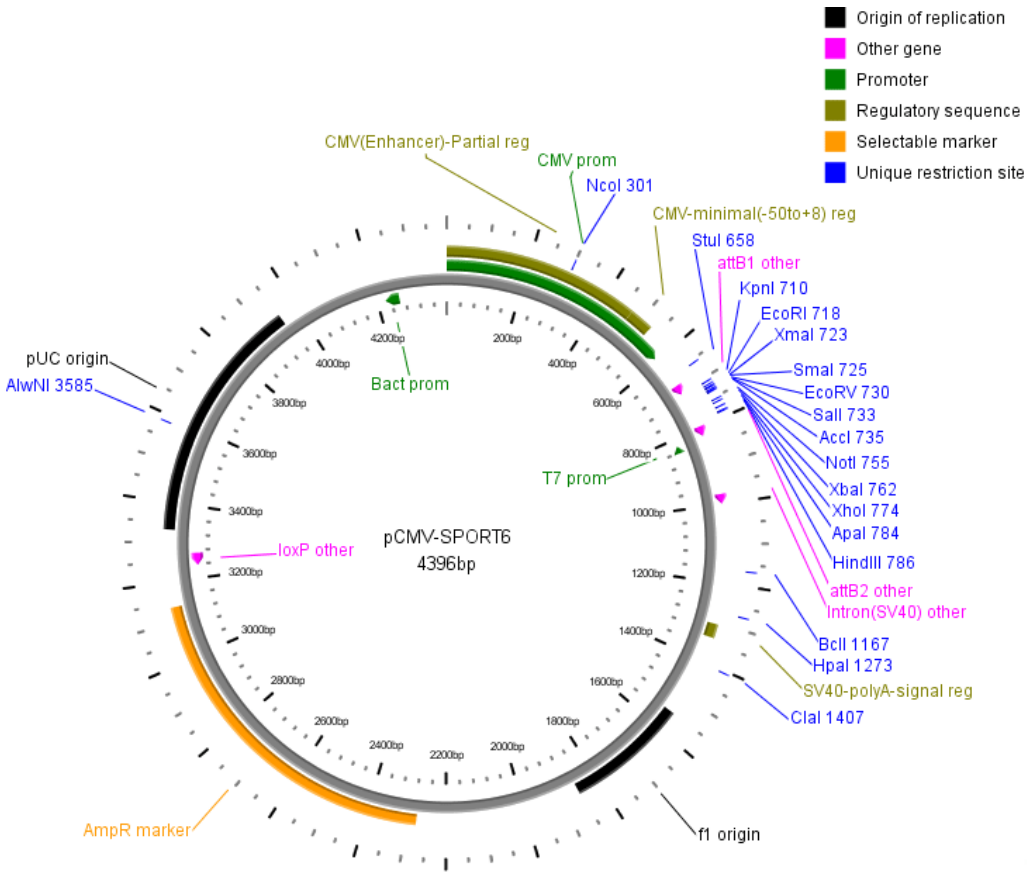


Figure 2.1 A schematic map of the pCMV-SPORT6 vector

The vector contains the FABP7 gene (1004bp; accession number NM_001446.3) between the 5' SalI and 3' NotI restriction sites. The complete plasmid size is 5400bp. The insert was removed using SalI and XhoI restriction enzymes creating a fragment of length of around 1000bp with the remaining vector fragment of 4353bp.

The glycerol stock was used to inoculate a Lysogeny broth (LB) agar plate (ddH_20, containing 10g/L of tryptone, 5g/L of yeast extract, 5g/L of NaCl and 15g of agar autoclaved, poured into sterile dishes and the allowed to set). Due to the ampicillin resistance gene on the plasmid, 100µg/mL ampicillin (Sigma-Aldrich) was added to the agar whilst still molten, to prevent contaminants, or untransformed *E.coli*, from growing. The agar plate was incubated overnight at 37^{o}C; the following day a single colony was selected to inoculate 5mL of LB (no agar) also containing 100µg/mL ampicillin. The broth was incubated overnight on a shaker at 200rpm. at 37^{o}C.

2.8.2 Plasmid purification

The overnight cultures to generate plasmid were purified using the PureYield Plasmid Miniprep System (Promega, Southampton, UK); it was used according to manufacturer's instructions. 3mL of culture was spun to pellet the cells; the culture media supernatant was removed and then the cells re-suspended 600µL of nuclease free water. The resuspension was then subjected to plasmid purification.

2.8.3 Generation of a control pCMV-SPORT6 vector

To generate a control pCMV-SPORT6 vector, 1µg of purified plasmid, containing the FABP7 gene, was digested using the restriction enzymes SalI and XhoI (Promega, Southampton, UK). XhoI was used instead of NotI as it has compatible ends with SalI and is only a few base pairs away; no important plasmid features were lost. Restriction digest took place at 4^{o}C overnight; the digest contained 16.3µL of ddH_2O. 2µL of restriction enzyme 10X buffer, 0.2µL BSA (1mg/mL), 1µg of DNA and 0.5µL restriction enzyme (10u/µL), (Promega, Southampton, UK) giving a final volume of 20µL.

The following morning 5µL of DNA loading buffer was added and the restriction digest was run on a 1% TAE buffer agarose gel at 100V for 30 minutes. Two bands were seen; a smaller band of around size 900-1000bp and a larger the size of around 4000-4500bp; these bands were the predicted size for the FABP7 gene and the plasmid backbone respectively. The larger 4000-4500bp band, containing the plasmid backbone, was extracted from the gel and purified using the wizard SV gel prep kit according to manufacturer's instructions. The purified DNA was then religated with T4 DNA ligase (Promega, Southampton, UK) due to the compatible ends. 0.5µg of DNA was incubated overnight with T4 ligase and its respective buffer, at 4°C. The control pCMV-SPORT6 plasmid was transformed into JM109 competent cells (Promega Southampton, UK) by heat shock. Bacterial cells were cultured in super optimal broth with catabolite repression (SOC) medium (supplied with JM109 cells) overnight on a shaker at 200rpm at 37^{o}C. The culture broth was then streaked and incubated on an LB plate with ampicillin as previously described. This ensured selection of only transformed JM109 bacterial cells. A single colony was then selected for growth in LB broth and then the control plasmid was then purified as described above.

2.8.4 Glycerol stock generation

Glycerol stocks of cultured bacteria; both the FABP7 and JM109 cells; were made by mixing 900µL of culture with 100µL of glycerol to give a 10% glycerol solution. The stocks were kept at -80°C until required for use. Further validation of plasmids took place as in chapter 5.

2.8.5 Transfection procedure optimisation

The day before transfection, cells were seeded as previously described into 6 well plates at a density of 0.5x10^{6} cell per well with complete growth medium. The following morning cells were serum starved in Optimem growth medium (Invitrogen, Paisley, UK) for two hours. During this

time the transfection solutions were prepared. Fugene HD (Promega, Southampton, UK) transfection reagent was allowed to warm up to room temperature. 0.5µg, 1µg, 2µg and 3µg of purified GFP plasmid (a kind gift from the University of Bristol) were tested with a 1:2 and 1:3 volume of Fugene HD. For example 0.5 µg was tested with 1µL or 1.5µL of Fugene HD per well. In line with this a plasmid alone well and a Fugene HD alone well were set up and treated the same as the other wells for use as a control to assess any effect of the 2 components in isolation. The plasmid was added to 100µL (volume per well) of Optimem and vortexed. An appropriate volume of Fugene HD (depending on the amount of plasmid and ratio tested) was then added to the tube without it being allowed to touch the sides. The transfection solution was then left to incubate for 15 minutes at room temperature. After this time, the solution was added drop-wise to the cells. After four hours the culture medium with transfection reagent was removed and replaced with complete growth medium. Forty-eight hours later, to allow maximal protein production, the amount of fluorescence from the GFP produced was assessed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences, Oxford, UK). This gave an indication of the optimal conditions. Toxicity of the transfection was noted by observing the cells under a microscope. Cells were removed from the wells with 0.25% Trypsin/0.53mM EDTA (Sigma-Aldrich, Dorset, UK) and the resuspended in 500µL of PBS to prevent phenol red interference. The reading was gated to included live cells and exclude debris; the FL1 channel was used to measure the percentage of cells fluorescing and judge the transfection efficiency. Propidium iodide (PI) staining was then used to judge the toxicity of the transfection conditions in addition to microscopic evaluation. PI solution was prepared by dissolving 10µg of PI per 1mL of PBS; the solution was stored at 4^{o}C in the dark. After fluorescence reading with the FL1 channel, 5µL of PI was added to the cell suspension and mixed by pipetting. The sample was passed through the flow cytometer and the level of PI staining was assessed using the FL2 channel. The final conditions chosen were 2µg of plasmid with a 1:3 ratio of FugeneHD per well of a 6 well tissue culture plate.

2.9 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay is a quantitative colourimetric assay that is used to observe cell viability and cytotoxicity. It does this by assessing mitochondrial metabolic activity. Twenty-four hours post transfection; BT-20 cells were seeded at a density of 6,000 cells per well of a 96 well plate. Six wells were seeded per condition; i.e. 6 wells of control plasmid transfection and 6 wells of FABP7 plasmid transfection. The following morning cells had 10µL of MTT (concentration 5mg/mL; Sigma Aldrich, Dorset, UK) added and incubated at 37°C in the dark for three hours. Medium was then removed and 25µL of Sorensons glycine buffer (0.1M glycine, 0.1M NaCl, adjusted to pH 10.5) was added to increase sensitivity; 200µL of DMSO was added and the plate incubated on a shaker for 10 minutes before reading at 570nm on a plate reader.

2.10 Oil red O staining

Oil red O is a fat soluble dye used to demonstrate triglycerides usually in frozen tissue sections. This adapted method was used to study whether there was any difference in fat accumulation in transfected BT-20 breast cancer cells. Cells were treated with FAs (section 2.3 onwards), the final doses for the FAs were chosen as follows palmitate 50µM, oleate 100µM, AA 10µM and DHA 10µM; these doses were chosen based on dose response results (section 1.10). Twenty-four hours later the cells were washed twice with PBS and then incubated in 10% formalin (10mL of formaldehyde with 90mL PBS) for 10 minutes. After 10 minutes the formalin was replaced with fresh solution and then incubated for one hour at room temperature. The cells were washed twice with ddH_2O and then with 60% isopropanol for five minutes. The isopropanol was removed and the cells allowed to dry completely. During this time the Oil red O working solution was made by mixing 6mL of stock Oil red O solution (0.35g Oil red O (Sigma-Aldrich, Dorset, UK) in 100mL isopropanol and filtered)) with 4mL of ddH_2O and letting the mixture stand for 20 minutes before

filtering. The working solution was added to the dry cells and incubated for 10 minutes. Following this the stain was removed and the cells washed four times with ddH_2O. The cells were then ready for imaging under an inverted microscope.

2.11 Statistical Analysis

The statistical software packages SPSS IBM statistics (version 20) and GraphPad Prism 5 were used for statistical analysis. The types of statistical test used for each experiment are detailed in the relevant results chapter sections.

3 FABP7 in triple negative breast cancer

3.1 Introduction

Triple negative (TN) breast cancers are defined by their lack of expression of the oestrogen receptors (ER) and progesterone receptors (PR) and human epidermal growth factor receptor 2 (Her2) and account for 10-24% of all breast cancers (Reis-Filho & Tutt, 2008; Tan *et al.*, 2009; Viale *et al.*, 2009). Notably, TN breast cancers share many overlapping characteristics with basal-like breast cancers (BLBC), in that the majority of TN breast cancers have elevated expression of high molecular weight cytokeratins and both have similar gene expression signatures (Dent *et al.*, 2007; Kreike *et al.*, 2007; Rakha *et al.*, 2007a; Tan *et al.*, 2008; Cheang *et al.*, 2008). TN tumours are predominantly high grade and aggressive cancers with poor prognosis and unlike hormone receptor and Her2 positive breast carcinomas, there are no targeted therapeutic regimes that have been shown to significantly improve survival (Carey *et al.*, 2010). The treatment of these tumours is therefore challenging and biomarker studies are required to better characterise these tumours with the aim of identifying improved therapeutic interventions. One biomarker that has been investigated to a limited extent and that is up-regulated in BLBC is FABP7. FABP7 is one of 9 FABPs that have roles in FA metabolism and transport. Studies have given conflicting evidence as to the role of FABP7 in breast cancer (Zhang *et al.*, 2010; Tang *et al.*, 2010); studies looking at FABP7 in other cancers such as melanoma and in gliomas have been more consistent in demonstrating that it is associated with poor prognosis (Liang *et al.*, 2006; Slipicevic *et al.*, 2008; Goto *et al.*, 2010).

A Malaysian TN breast cancer cohort was investigated for FABP7 expression using Immunohistochemistry (IHC). Assessment of FABP7 expression in clinical samples will investigate further its important in BLBC/TN breast cancer and will provide evidence as to whether FABP7 is

associated with good or poor prognosis. FAS is an enzyme that is responsible for the conversion of malonylCoA to palmitate (figure 1.12); FAS is up-regulated in many cancers including those of the breast (Alo' *et al.*, 1996; Shurbaji *et al.*, 1996; Rashid *et al.*, 1997; Kuhajda, 2000). It was hypothesised that FAS expression may correlate with FABP7 expression due to the evidence of the interaction of FABP7 with palmitate.

Breast cancer cell lines are invaluable in fully examining functional roles that FABP7 may be playing in breast cancer pathology. However interestingly despite other studies demonstrating expression of FABP7 in clinical cases of breast cancer, it has proven difficult to detect FABP7 in *in vitro* breast cancer cell lines. One study knocked down FABP7 in MDA-MB-435 cells, using these cells as a breast cancer model (Liu *et al.*, 2012), however there is dispute as to whether the MDA-MD-435 cell line is of breast or melanoma origin (Rae *et al.*, 2007; Lacroix, 2009). Consequently the aim of the current study was to develop a reproducible method that can be used to detect FABP7 protein in breast cancer cell lines lysates. This method, alongside the investigation of FABP7 mRNA and protein expression in patient samples and cell lines, will aid in the future investigation of FABP7 in breast cancer.

3.2 Aims and hypothesis

3.2.1 Aims

i) To quantify FABP7 mRNA and protein in breast cell lines in order to choose an appropriate model for further investigation.

ii) To assess FABP7 IHC staining in a cohort of Malaysian TN breast cancer cases and look at associations with basal-like phenotype and clinicopathological features.

3.2.2 Hypothesis

i) FABP7 expression is associated with the basal-like phenotype, FAS expression and clinicopathological features and is therefore of prognostic value in TN breast cancer.

3.3 Materials and Methods

3.3.1 Cell lines used

The breast cancer cell lines used were BT-20, MDA-MB-231, MDA-MB-453, MDA-MB-361, MCF-7, T47D, SKBR3 and HS578T; the normal breast cell line MCF10A was also used (ATCC, UK). The glioma cell line U251 (ATCC, UK) was also used for this work. Cell lines were grown according to the supplier's instructions either as detailed previously in section 2.2 or cultured by collaborators at the University of Bristol, before lysis.

3.3.2 Positive controls for western blotting

The glioma cell line U251 was cultured for use as a FABP7 positive control as studies have confirmed it expresses FABP7 mRNA and protein at high levels (Mita *et al*., 2010). A full length recombinant FABP7 peptide (Abcam, Cambridge, UK), was also used as positive control for the detection of FABP7 protein.

3.3.3 Antibody optimisation for western blotting

A goat polyclonal anti-FABP7 antibody (R&D Systems, USA) and the G-13 anti-FABP7 antibody (Santa Cruz Biotechnology, UK) were compared. The primary antibodies were tested at dilutions between 1:200 and 1:1000, with antibody binding carried out overnight. The positive control full length FABP7 peptide (Abcam, UK) and the FABP7 positive glioma cell line U251 were included. The anti-goat secondary antibody (Sigma-Aldrich, UK) was tested at dilutions between 1:1000 and 1:2000 with antibody binding being carried out for one hour at room temperature.

3.3.4 Blocking reagent and antibody diluent optimisation for western blotting

The effect of blocking concentration was assessed by utilising two concentrations of blocking reagent, with blocking carried out for 1hr. 5% BSA-TBS-T and 3% BSA-TBS-T were utilised for both the initial blocking step and as the antibody diluent.

3.3.5 Transfer time for western blotting

The effect of transfer time on efficiency of protein detection was assessed by transferring for one hour or 1.5 hours at 100V onto Hybond-P PVDF (GE Healthcare Life Sciences, USA) membrane using the Mini Trans-Blot apparatus (Bio-Rad, UK) according to manufacturer's instructions for wet blotting.

3.3.6 Protein loading for acrylamide gel electrophoresis

The threshold of FABP7 detection, by western blotting, in breast cancer cell lines was investigated by loading increasing amounts of total protein (50µg-100µg). In addition reducing versus non-reducing protein loading conditions were investigated. Non-reducing buffer comprised of ddH_20 (37ml), 0.5M Tris-HCl (10ml), Glycerol (8ml), 10% SDS (16ml) and 0.05% Bromophenol Blue (5ml).

3.3.7 Cell lysis optimisation for protein extraction

Protein lysates were prepared in a number of ways to investigate the effect on FABP7 protein detection; 10µL each of phosphatase inhibitor II and protease inhibitor cocktails (Sigma-Aldrich, Dorset, UK) were added per 1mL of each lysis buffer. Standard cell lysis was carried out using lysis buffer prepared in house (section 2.4); briefly cells were washed twice with PBS and then lysis buffer applied directly to the culture vessel. This was incubated for 30 minutes at 4^oC with gentle agitation. The lysate was then collected, clarified by centrifugation for 15 minutes at 4^oC (11200

g) and the supernatant collected. Prior to gel loading, samples were quantified, reduced and denatured by heating at 99^{o}C for 5 minutes with an equal volume of Laemmli x2 concentrate sample buffer (Sigma-Aldrich, UK).

RIPA buffer lysis was compared as it has been found to be more appropriate for extracting nuclear proteins. Briefly cells were washed twice with PBS and then lysis buffer applied directly to the culture vessel. This was incubated for 30 minutes at 4^{o}C with gentle agitation. The lysate was then collected, clarified by centrifugation for 15 minutes at 4^{o}C (11200 g) and the supernatant collected.

The final alternative method of lysis investigated was using Laemmli x2 concentrate sample buffer (sample buffer) (Sigma-Aldrich, UK). One study found that Laemmli sample buffer lysis gave better protein yields from tissues that had been formalin fixed and paraffin embedded (Nirmalan *et al*., 2009). Direct lysis provided the benefit of more concentrated lysate production, thus enabling larger amounts of total protein to be loaded per well, which in turn would increase the ability to detect proteins of low abundance. Briefly cells were washed twice in PBS and then lysed directly in the culture vessel by addition of sample buffer. The flask was incubated at 4^{o}C for 30 minutes with gentle agitation, followed by collection and sonication in a water bath for 15 minutes to ensure shearing of DNA, thereby reducing the viscosity of the samples. Prior to gel loading, samples lysed in in-house lysis buffer or RIPA buffer, were heated at 99^{o}C for 5 minutes with an equal volume of sample buffer. Samples lysed in sample buffer were heated at 99^{o}C for 5 minutes and loaded immediately to further minimise viscosity issues and aid loading.

3.3.8 Protein Quantification

The amount of total protein present in the cell lysates was determined using a BCA protein assay (Pierce, USA), except for samples lysed directly in Laemmli sample buffer, where the 660nm

protein assay kit (Pierce, USA) was used both according to manufacturer's instructions (section 2.5).

3.3.9 Acrylamide gel electrophoresis optimisation

Optimisation of electrophoresis was achieved by comparison of protein separation on 12% gels prepared in house (section 2.5) and pre-cast Mini PROTEAN TGX 4-20% gradient gels (Bio-Rad, UK). Electrophoresis was carried out using the Mini-PROTEAN Tetra system (Bio-Rad, UK).

3.3.10 Protein Visualisation

Proteins were visualised using SuperSignal West Femto Chemiluminescent Substrate kit (Pierce, USA) and imaged using a ChemiDocIt system and VisionWorksLS software (UVP, UK), with β-Actin used as a control for loading. The membrane was cut as described in section 2.5.5 before probing with primary antibodies. In addition positive controls were sometimes cut from the same membrane for imaging separately to prevent any interference with protein detection in the breast samples.

Protein was semi-quantified using densitometry by measuring band intensity in ImageJ. Briefly the region of interest tool was selected and exactly the same size box was drawn around each band; the program then generated arbitrary numbers based on the intensity of each band. B-actin was checked to ensure even sample loading

3.3.11 RNA extraction and PCR

For this work the Bioline kit was used to extract RNA as in section 2.6.1; reverse transcription and qPCR then took place as in 2.7.1. For this work 20 cases had tissue blocks available; tissue curls

were taken from all 20 to investigate the relationship between FABP7 expression detected by IHC and FABP7 mRNA levels in corresponding samples. RNA extraction from the tissue took place as in section 2.6.3.

3.3.12 Clinical cases of breast cancer and immunohistochemistry

Tissue samples from breast cancer patients reported as being negative for ER, PR and Her2 and diagnosed between 2004 and 2009 at the University of Malaya Medical Centre, were used in this study. All tissues had been fixed in 10% neutral buffered formalin between six and 72 hours, and processed to paraffin wax blocks, from which sections were cut at 3µm thickness on a rotary microtome and mounted onto Tissue Tek Plus glass slides to ensure maximum adhesion.

The TN phenotype was confirmed by using IHC for ER PR and Her2 (section 2.1) and positive cases excluded from further analysis. FABP7, cytokeratins 5/6 and cytokeratin 14 were tested for by IHC as described in section 2.1. A total of 101 were cases identified as having adequate invasive tumour tissue for evaluation and were negative after ER, PR and Her2 testing.

3.3.13 Assessment of immunohistochemistry

Staining was assessed as described in section 2.1.9. Cases that were not possible to interpret or did not have sufficient positive control staining were omitted from analysis.

3.3.14 Assessment of Tumour Grade and Stage

Grading was according to the modified Bloom and Richardson criteria (Elston & Ellis, 1991). All slides were reviewed and re-graded for this study by clinical pathologists from the Department of Pathology, University of Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia. Clinical data

on patient age, ethnicity and stage, were extracted from the UMMC database for this series of cases.

3.3.15 Statistical analysis

Continuous variables (age) were described using medians and compared using the Mann-Whitney U test, while categorical variables (ethnicity, stage, grade, FABP7 score, CK5/6 expression and CK14 expression) were expressed as proportions and compared using either Chi square test or Fisher's Exact test. A Kaplan Meier survival analysis took place on survival data from UMMC.

3.4 Results

3.4.1 Antibody selection, optimisation and assessment of specificity

At the time of this study there were limited available antibodies for FABP7 that were validated for western blotting. Two commercially available anti-FABP7 primary antibodies were tested; the goat anti-FABP7 antibody (R&D systems) and the goat anti-FABP7 antibody, clone G13 (Santa Cruz Biotechnology). The antibody diluent was 5% milk powder in TBS-T unless otherwise stated. The R&D systems primary antibody and HRP labelled anti-goat secondary antibody (Sigma-Aldrich) dilutions were tested in a variety of combinations on blots of 20ng and 50ng of FABP7 full length peptide and 30μg lysate from U251 cells. The secondary antibody dilution, 1:1000, led to the development of background as in figure 3.1 A and C. The primary antibody dilution 1:800 did not have sufficient sensitivity for the detection of the 20ng of peptide (figure 3.1 C and D). The combination of 1:500 primary antibody with 1:2000 secondary antibody gave the sharpest, bands whilst achieving the best threshold of detection (figure 3.1 B). As a result this combination was selected to facilitate further optimisation.

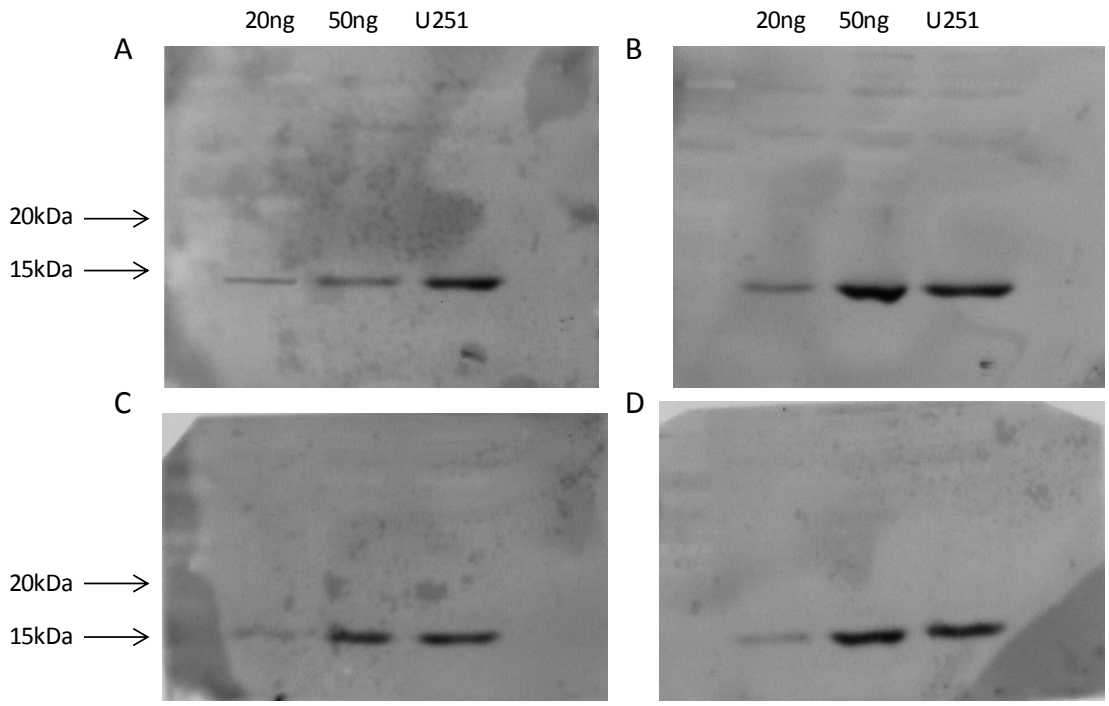


Figure 3.1 The optimisation of primary and secondary antibody dilution

The FABP7 peptide and U251 lysate were separated by SDS-PAGE and then blotted on to PVDF membrane. The membrane was cut in to 4 pieces and each piece incubated in a different combination of primary and secondary antibody dilution. A) primary 1:500, secondary 1:1500. B) primary 1:500, secondary 1:2000. C) primary 1:800, secondary 1:1500. D) primary 1:800, secondary 1:2000. The final combination chosen was B as it gave the least background and greatest sensitivity.

For comparison of the two available antibodies the dilution of the G13 anti-FABP7 antibody was optimised and results compared. The G13 antibody was tested at dilutions 1:200 (Figure 3.2B), 1:500 (Figure 3.2C) and 1:1000 (Figure 3.2D) on 30µg of protein lysate from U251 cells. The secondary antibody was used at a dilution of 1:2000. The G13 antibody consistently produced higher background and was less sensitive than the R & D antibody (figure 3.2A). However both antibodies identified single molecular weight products at the expected molecular weight. Although the R&D antibody outperformed its counterpart it was essential to ensure specificity. This was achieved using the commercially available blocking peptide for Santa Cruz.

Briefly the FABP7 blocking peptide was used at five times the concentration of the FABP7 & antibody from Santa Cruz, as recommended in the manufacturer's instructions. The antibody and blocking peptide were incubated together for two hours at room temperature. Following incubation the antibody-blocking peptide solution was added to the diluent (5% milk powder in TBS-T) to achieve the desired 1:200 dilution of G13 primary antibody. This blot was analysed alongside the R&D FABP7 antibody as a method control (Figure 3.2 A).

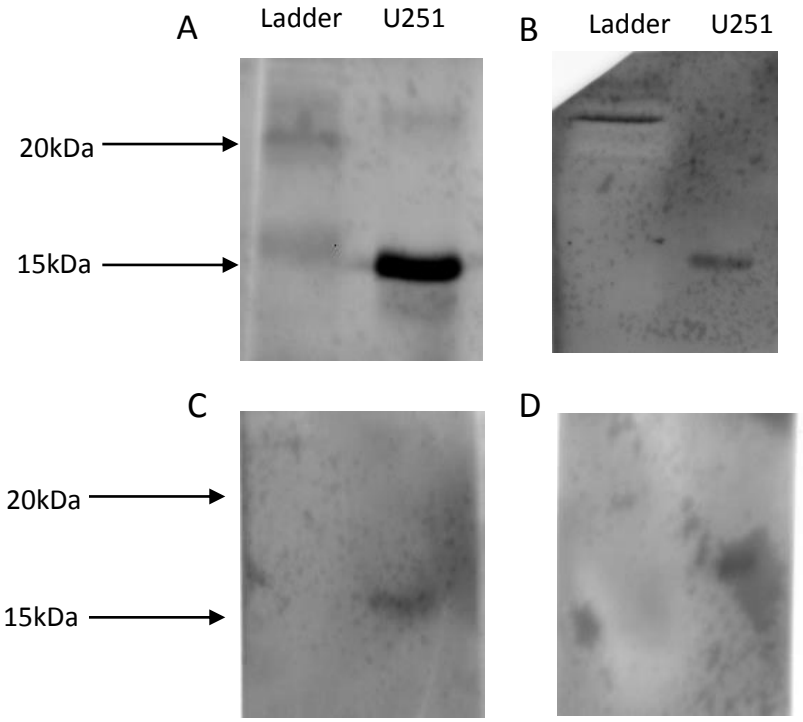


Figure 3.2 Comparison of two different FABP7 antibodies

The sensitivity and specificity of the SC FABP7 antibody was investigated by testing a variety of dilutions: B) 1:200, C) 1:500 and D) 1:1000, against the optimised R&D antibody shown in A. The bands were not as clearly defined and there was more background with the SC antibody.

3.4.2 Optimisation of Blocking Agent

Blocking reagent can influence the sensitivity and linear dynamic range of any given protein detected in a western blot protocol. These effects depend on the unique specific antigen-antibody pairing characteristics and thus need to be evaluated on an individual basis. To test whether the choice of blocking reagent significantly affected the detection of FABP7, membranes with FABP7 recombinant peptide and U251 lysate were blocked in either 5% milk (figure 3.3A) powder or 3% BSA (Figure 3.3B). The BSA led to increased background. Since the milk powder blocking agent provided the highest signal-to noise ratio, 5% milk powder in TBS-T was used for all subsequent experiments.

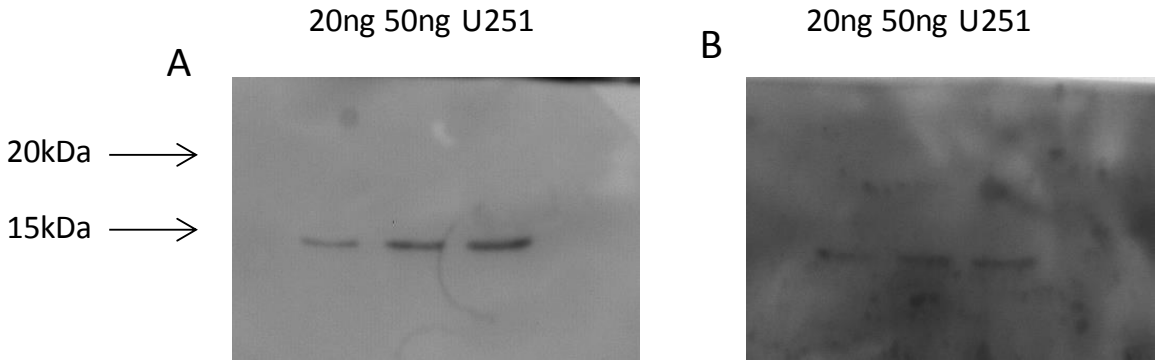


Figure 3.3 The optimisation of blocking reagent

FABP7 full length recombinant peptide and a U251 protein lysate were loaded in replicate form onto the gel and separated and blotted as described previously. The resulting membrane was divided for uniformity of samples and blocked in A) 5% milk or B) 3% BSA respectively. The primary and secondary antibodies were diluted in their respective blocking solutions. The highest signal-to-noise ratio was achieved using milk.

3.4.3 Comparison of reducing and non-reducing conditions in protein preparation

Antibody-epitope binding is greatly affected by the conformational structure of the protein of interest. For instance epitopes may be lost or gained if the protein is reduced or denatured when disulphide bonds are broken and the protein "linearized". There was no information on the R&D antibody data sheet about the conditions to which the protein lysates should be subjected. For these reasons reducing and non-reducing conditions during protein lysate preparation were tested to investigate whether FABP7 protein detection was improved using either condition. Non-reducing buffer comprised of ddH20 (37mL), 0.5M Tris-HCl (10mL), Glycerol (8mL), 10% SDS (16mL) and 0.05% Bromophenol Blue (5mL). As expected non-reducing conditions (figure 3.4) resulted in the detected protein band, running at a higher molecular weight than predicted according to the manufacturer's data. However only one band was still detected and this was not deemed to be the result of dimerization or aggregation based on molecular weight analysis. There was also no change in band intensity as a result of non-reducing conditions showing that the R&D anti-FABP7 antibody epitopes were unchanged. This demonstrates that the protein lysates should be subjected to reducing conditions in order to linearize them so they separate to the predicted size during gel electrophoresis. In addition it is possible that proteins aggregated and were less able to migrate through the gel. Studies have consistently shown that the reducing conditions affect protein migration profiles; non-reducing conditions lead to the protein being detected at a higher than predicted molecular weight (Routledge *et al.,* 1987) and illustrated the importance in choice of reducing condition when looking at dimers, monomers or aggregates of protein since dimers or multimers would be lost in reducing conditions (MacPhee, 2010; Lui *et al*., 2006). For these reasons reducing conditions were used in subsequent protein lysate preparation.

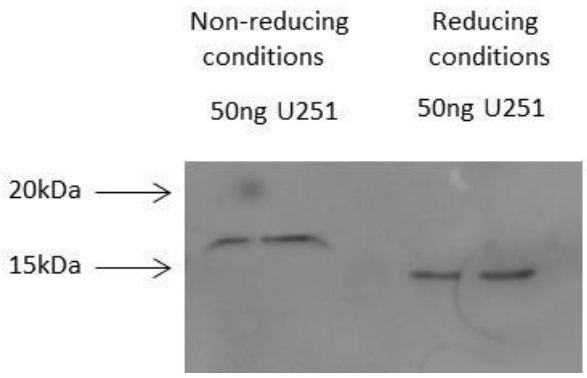


Figure 3.4 The optimisation of blocking reagent and sample reducing conditions

FABP7 full length recombinant peptide and a U251 protein lysate were subjected to reducing or non-reducing conditions and then SDS-PAGE and western blotting took place as described previously. The non-reducing conditions lead to the detected protein band running at a higher molecular weight than predicted.

3.4.4 Detection of FABP7 protein in breast cell-line lysates

With the main elements of the protocol optimised, a western blot was carried out with these optimised conditions on a panel of breast cell-line proteins lysates. The optimised conditions were as follows: Transfer 1 hour, blocking reagent and antibody diluents 5% milk, primary antibody R&D 1:500 and secondary antibody 1:2000. B-actin was used as a loading control. The resulting blot is shown in figure 3.6. Breast cell-line lysates were loaded initially at 50µg but FABP7 protein was not detected (results not shown). The amount of protein was increased to 100µg (figure 3.6). FABP7 was detected in 30µg of U251 lysate and the 50ng of FABP7 recombinant peptide was successfully detected, however FABP7 was not detected in the breast cell-lines under these conditions.

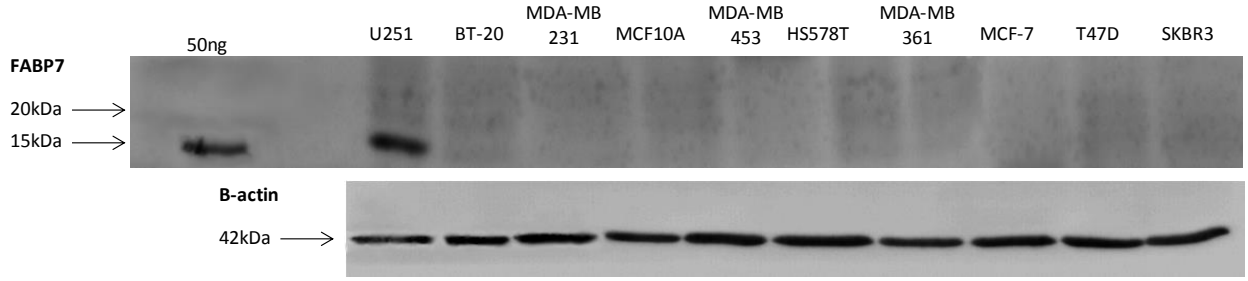


Figure 3.5 The detection of FABP7 in breast cancer protein lysates with conditions so far

Breast cell lines were lysed in standard lysis buffer and 100µg of each lysate prepared and subjected to western blotting as already described. The positive controls, recombinant FABP7 peptide (50ng) and U251 protein lysate were successfully detected and the loading control β-actin was even throughout the breast cell lines. FABP7 protein was not detected in the breast cancer cell lines.

3.4.5 Cell lysis optimisation

It was surprising, given the evidence that FABP7 protein is detected in breast cancer tissues using immunohistochemistry, that there was no FABP7 detected in any of the breast cell lines tested. A number of hypotheses for this were considered including the possibility of FABP7 being present in very low abundance or problems resulting from incomplete lysis and or solubilisation of cellular proteins. In order to ensure that the latter scenario was not hindering detection of FABP7 a variety of different protein extraction methods were evaluated using the positive control cell line U251. The subsequent U251 lysates were then quantified as described previously and seperated on a 12% acrlaymide gel. Transfer time, blocking and antibody dilutions were as before. The U251 cells lysed in sample buffer resulted in the strongest FABP7 band (figure 3.7).

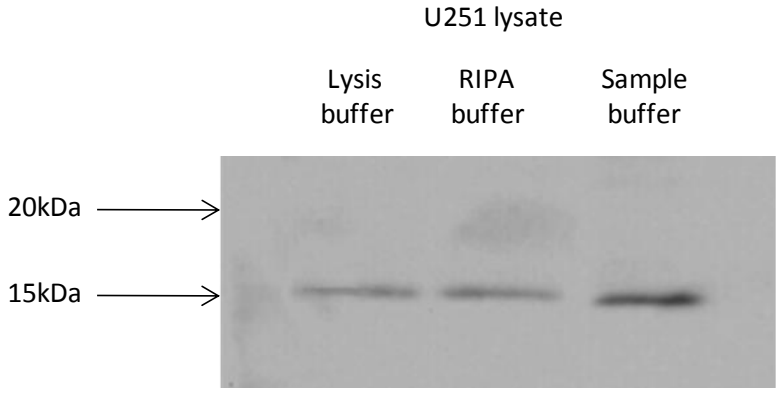


Figure 3.6 Lysis buffer optimisation

U251 cells were lysed in either standard lysis buffer, RIPA buffer or sample buffer and the subjected to SDS-PAGE on either 12% gel. Blotting and antibody incubations took places as previously optimised. The sample buffer gave the strongest band out of the lysis buffers tested.

3.4.6 Acrylamide gel electrophoresis optimisation

To investigate whether the type of gel used further improved detection of FABP7 a separation comparison was made using 12% gels prepared in house and pre-cast Mini PROTEAN TGX 4-20% gradient gels (Bio-Rad, UK). Gradient gels provided the benefit of increasing percentage up to 20% acrylamide, which would subsequently provide improved band sharpness for proteins such as FABP7 which have a very low molecular weight. Although sample buffer had consistently enabled the best signal detection, it was hypothesised that there may be a difference in protein migration between the gels depending on the lysis buffer used and consequently all lysis buffers were evaluated. The results are in figure 3.7; the gradient gel resulted in the strongest bands for all lysis buffers used. Following these results the breast cell lines were lysed in sample buffer and then subjected to electrophoresis on a gradient gel.

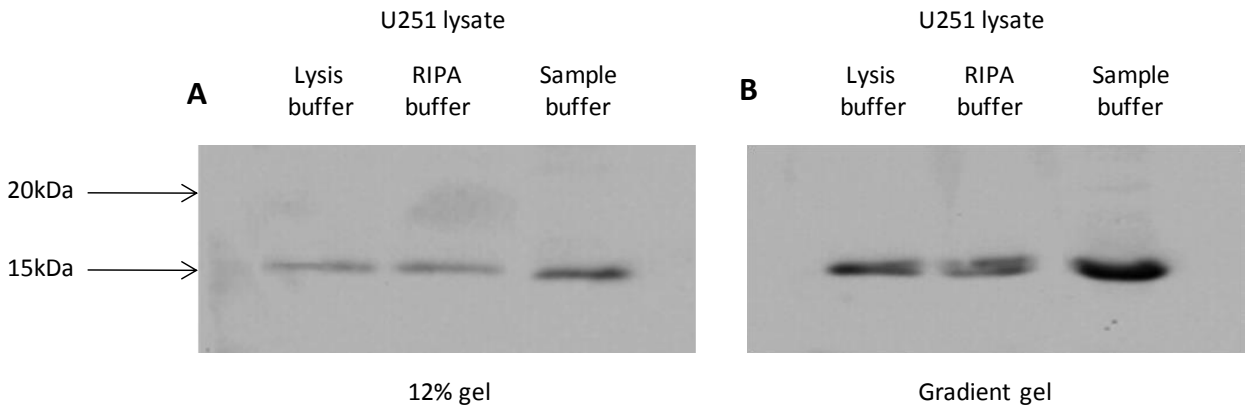


Figure 3.7 The differences in lysis buffer and choice of acrylamide gel.

U251 cells were lysed in either standard lysis buffer, RIPA buffer or sample buffer and the subjected to SDS-PAGE on either A) an in house 12% gel or B) a precast 4-20% gradient gel. Blotting and antibody incubations took places as previously optimised. The Sample buffer gave the strongest band out of the lysis buffers tested. The gradient gel lead to higher intensity bands overall than the 12% gel.

3.4.7 FABP7 protein and mRNA expression in a panel of breast cancer cell lines

A western blot, with the optimised conditions using sample buffer (x2 Laemmli sample buffer) for lysis and a gradient gel, was carried out with the available breast cancer cell lines (Figure 3.8 A). BT-20 cells expressed the most FABP7 protein whilst MCF10A and HS587T cells expressed the least. In addition, BT-20 cells also had the highest expression of FABP7 mRNA expression in the cell lines tested (Figure 3.9). MDA-MB-453 and MCF10A cells had the lowest mRNA expression.

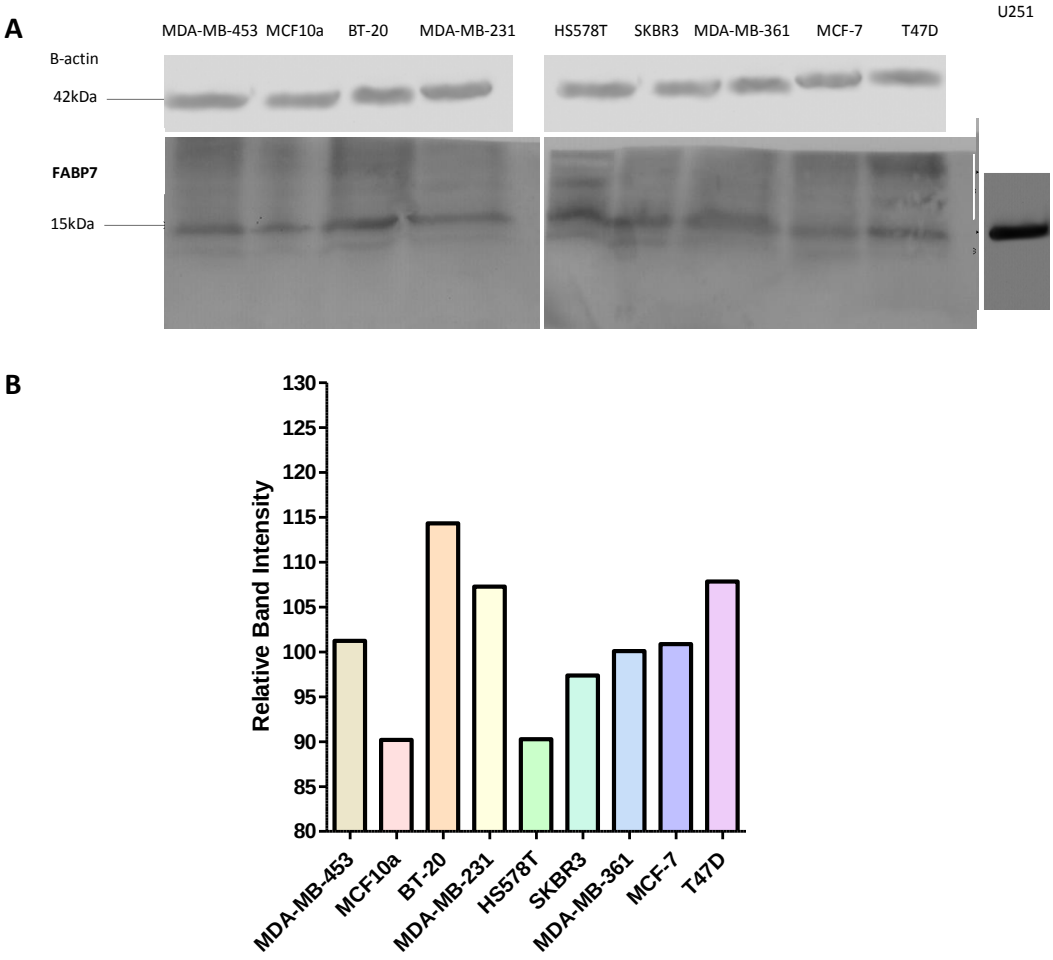


Figure 3.8 FABP7 protein expression in a panel of breast cancer cell lines

A: The resulting membrane image of FABP7 detection in breast cancer cell lines. The positive U251 control was imaged separately. B: The relative band intensity of the protein bands in A. This was interpreted using Image J software. BT-20 cells expressed the most protein in the panel. MCF10A and HS578T expressed the least. The results shown are representative of 3 independent experiments with the relative band intensities representing only the membrane in A.

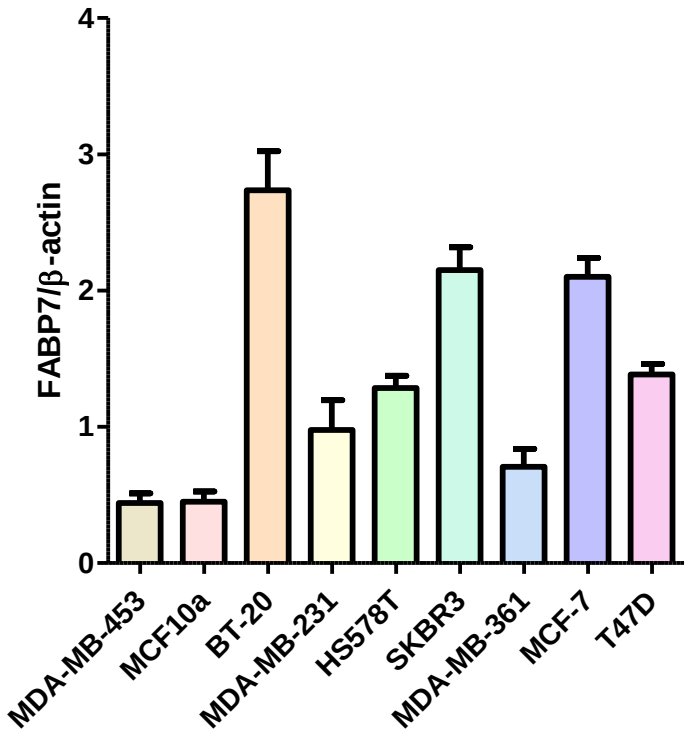


Figure 3.9 FABP7 mRNA expression in a panel of breast cancer cell lines

RNA was extracted from the breast cells and subjected to reverse transcription and then qPCR with β-actin as a reference gene. BT-20 cells had the highest relative expression of FABP7 mRNA followed by SKBR3 and MDA-MB-361 cells. MDA-MB-453 and MCF10A cell had the lowest expression of FABP7 mRNA. Results shown represent the mean of 3 independent experiments carried out in triplicate ±SEM.

3.4.8 FABP7 expression in patient samples

FABP7 staining was localised to the cytoplasm and nucleus of cancer cells with some cases and areas of tumours exhibiting areas of only cytoplasmic staining (figure 3.10). Staining intensity ranged from weak to intense (figure 3.11) and normal breast ducts and adipose tissue served as internal positive controls (figure 3.12). Of the cases available, 99 were assessable for FABP7. Twenty cases (20.2%) had complete absence of FABP7 staining; 38.3% of cases had intermediate-weak staining (score of 2-6) and 41.1% of cases exhibited strong staining (a score of 7 or 8) (table 3.1).

		Frequency	Percent
FABP7 score	0	20	20.2
	2	1	1.0
	3	4	4.0
	4	11	11.1
	5	13	13.1
	6	9	9.1
	7	11	11.1
	8	30	30.3
	Total	99	100.0

Table 3.1 Frequencies of FABP7 expression in a TN breast cancer cohort

Immunohistochemistry for FABP7 was carried out on TN breast cancer cases. There were 20.2% of cases that had complete absence of FABP7 staining. Intermediate-weak staining (score of 2-6) was present in 38.3%; the remaining cases (41.1%) had strong FABP7 staining (score of 7-8)

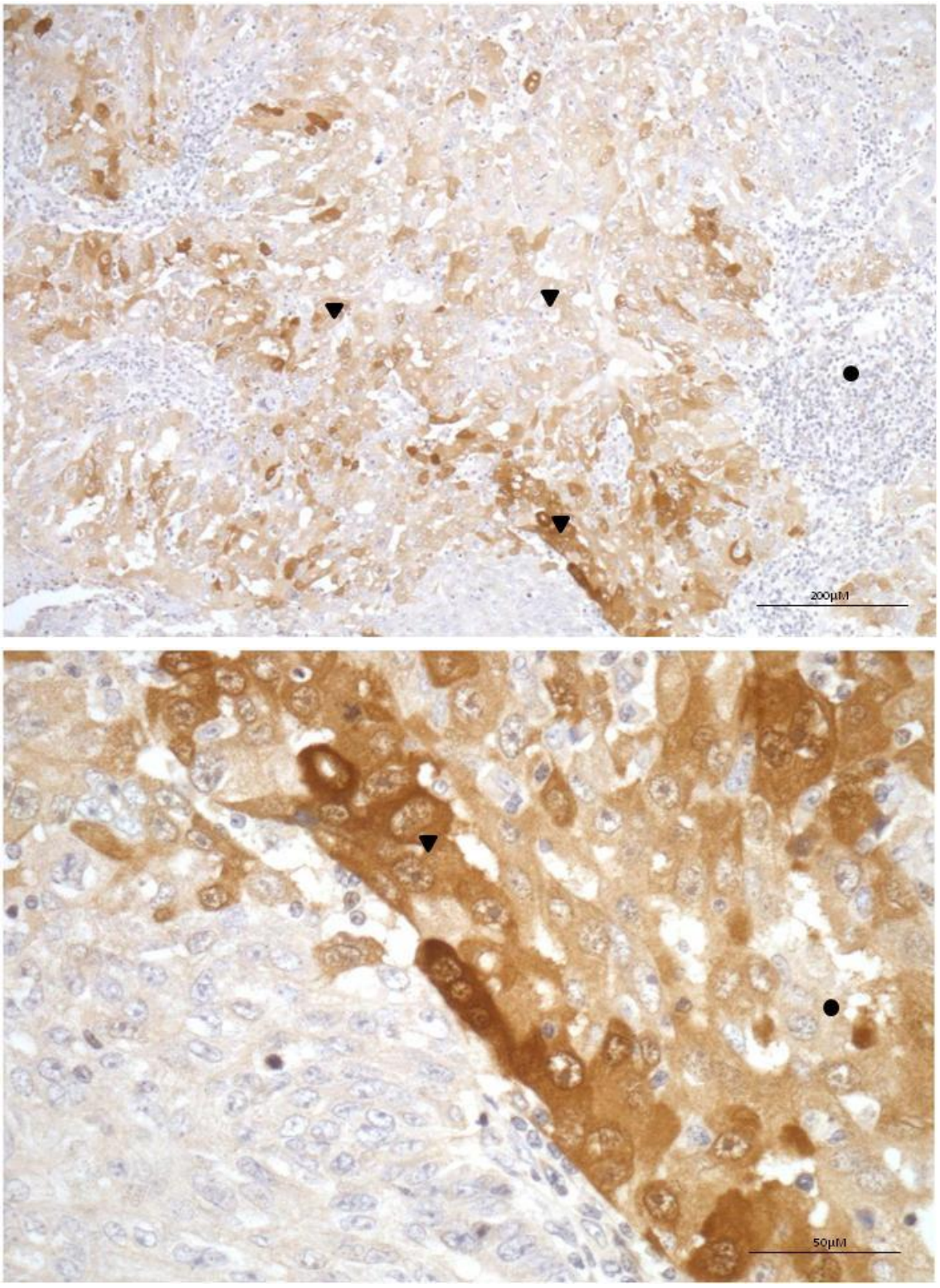
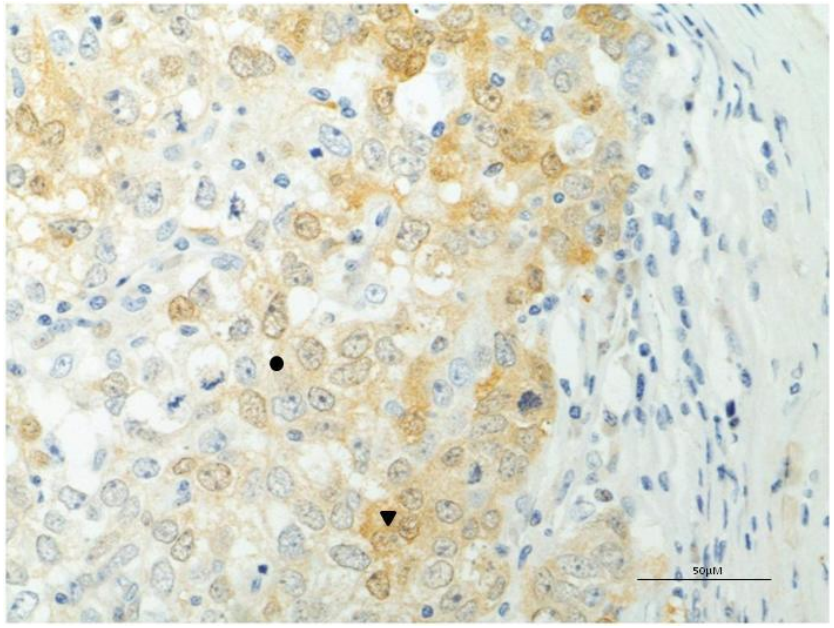
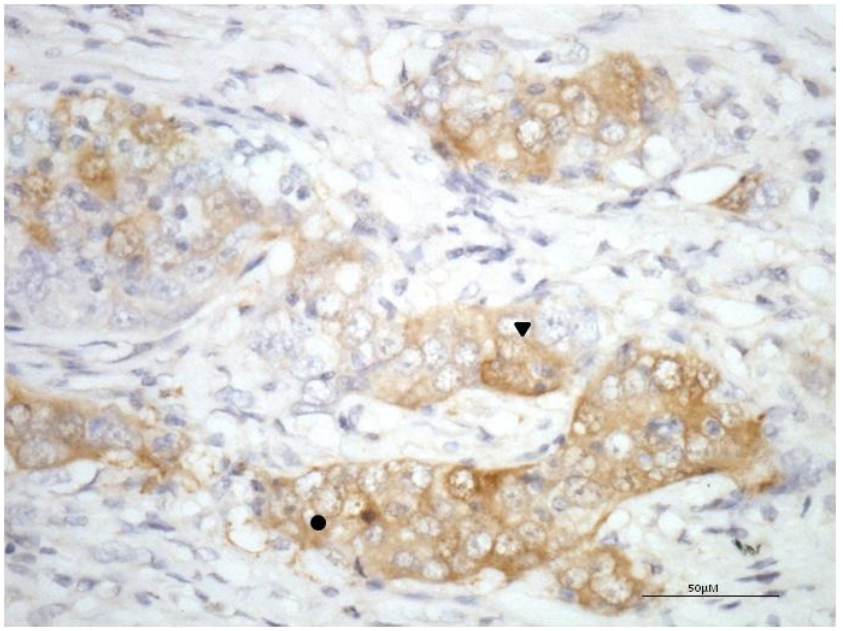


Figure 3.10 Patterns of FABP7 IHC staining in a triple negative, invasive breast ductal carcinoma (IDC) case.

Top: x10 "patchwork" pattern of FABP7 staining, areas of weak to strong staining of the IDC ◄ compared to negative staining of surrounding stromal tissue ●. Bottom: The same case at x40, showing cells with nuclear and cytoplasmic localisation ◄ and cytoplasmic only localisation of FABP7 ●.





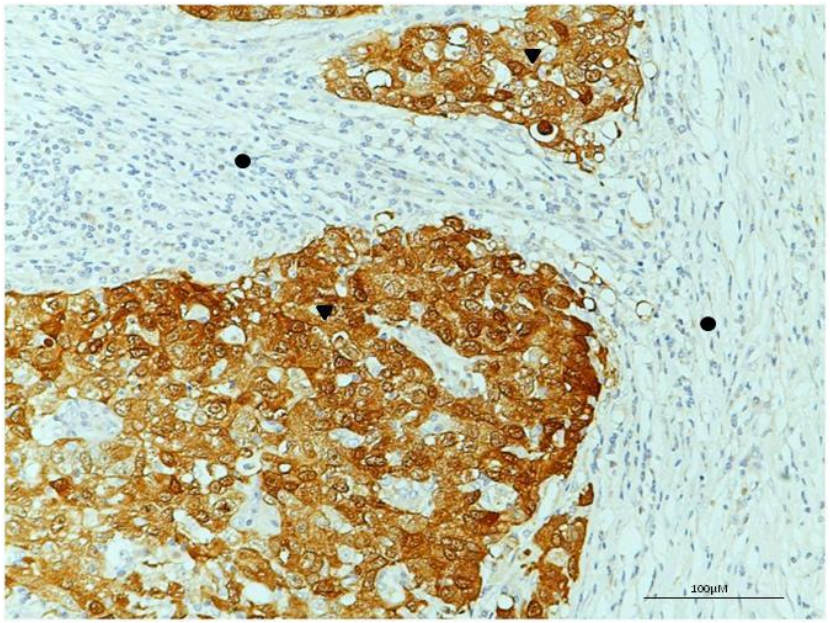


Figure 3.11 FABP7 IHC staining intensities in 3 triple negative IDC cases

Top left: x40 Weak FABP7 staining localised to both the nucleus ● and cytoplasm ◄ of cells in the IDC. Top right: x40 Intermediate-weak FABP7 staining, predominantly cytoplasmic ● but some nuclear localisation ◄. Bottom: x20 Strong FABP7 staining of the IDC ◄ compared to negative staining of the surrounding stromal tissue ●.

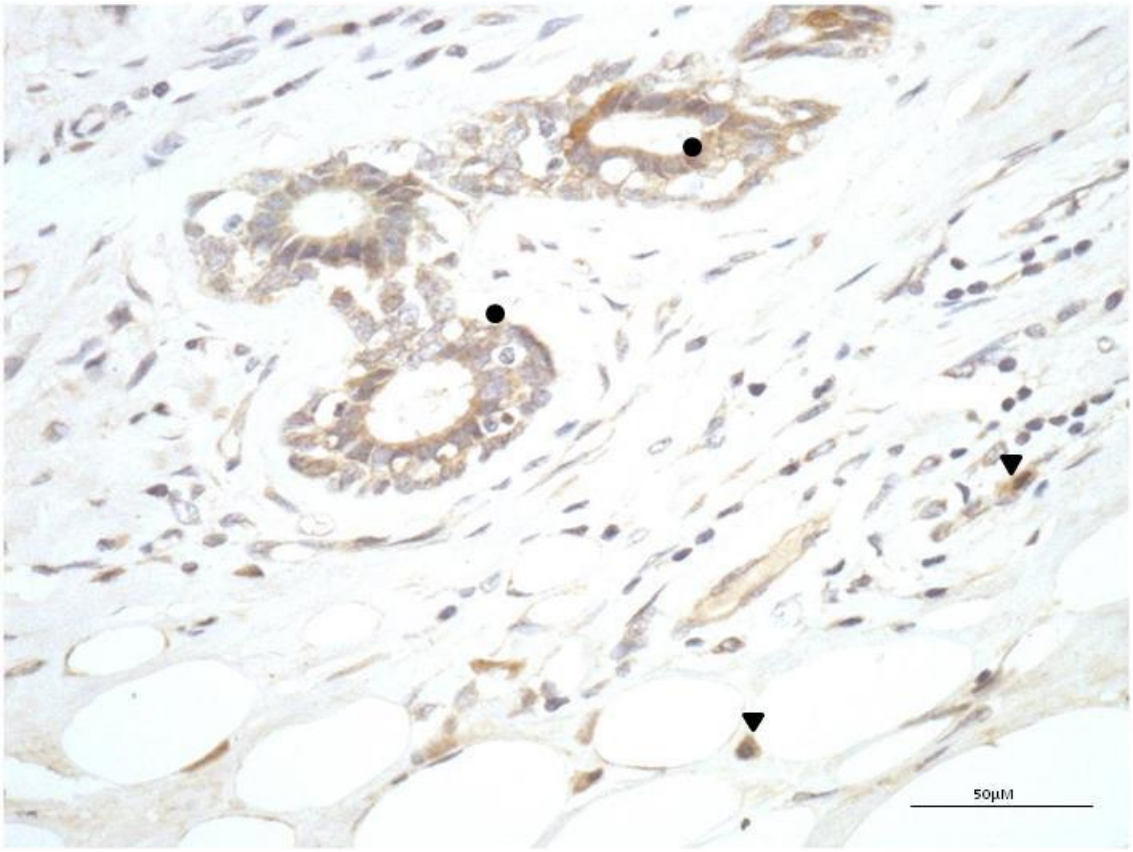


Figure 3.12 FABP7 IHC staining in normal ducts and adipose tissue

X40 Staining of FABP7 with IHC in normal ducts ● and adipose tissue ◄ served as internal positive controls.

3.4.9 Use of a FABP7 blocking peptide eliminated positive staining

A second FABP7 antibody (clone G13, Santa Cruz Biotechnology, UK) was used to validate the specificity of the staining due to the availability of a blocking peptide. The results are in figure 3.13. A strong FABP7 positive case was selected from the cases stained with the R&D antibody and staining repeated with the anti-FABP7 clone G13 antibody. The addition of the blocking peptide with the anti-FABP7 clone G13 antibody (section 2.1.6.3) eliminated the staining.

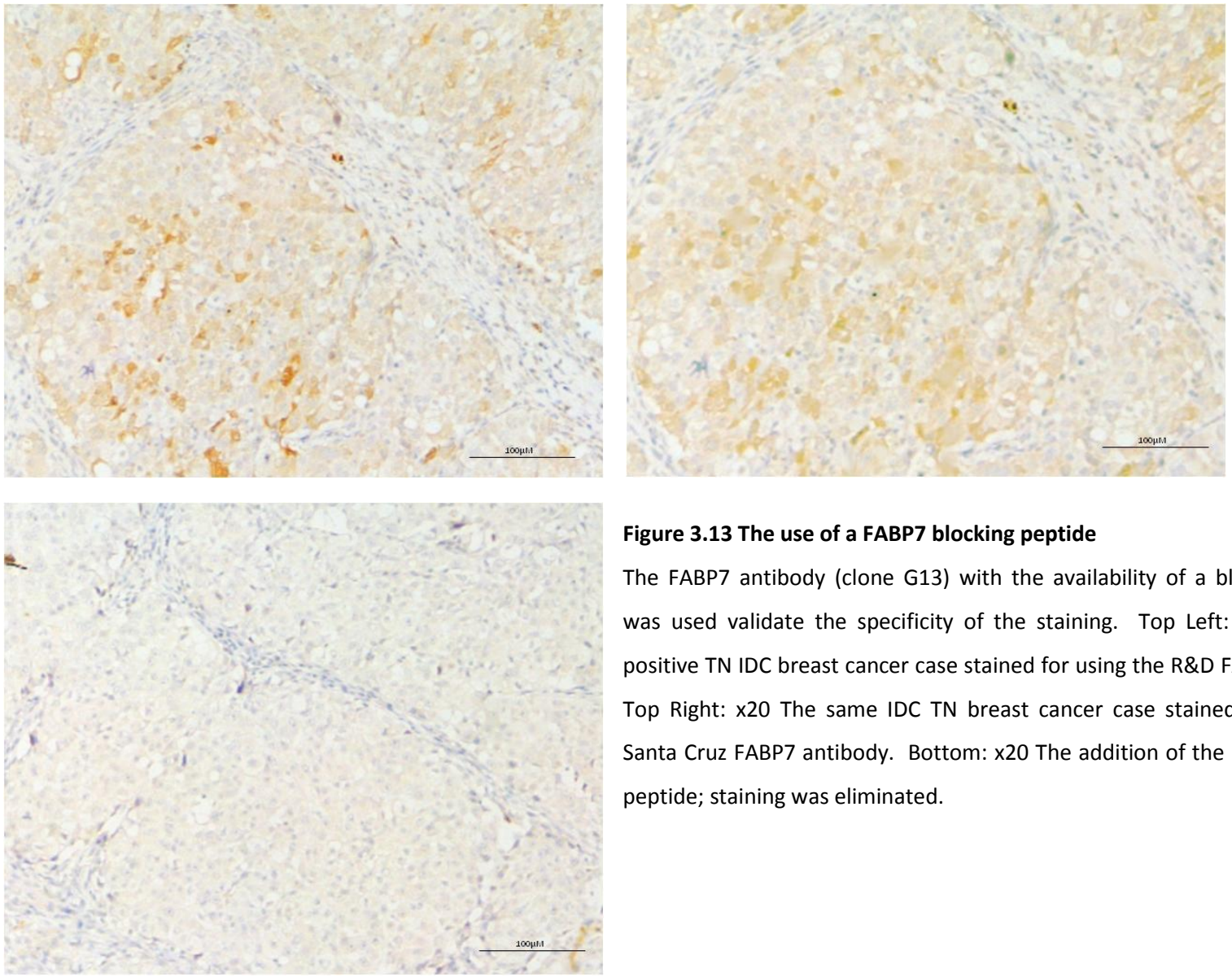


Figure 3.13 The use of a FABP7 blocking peptide

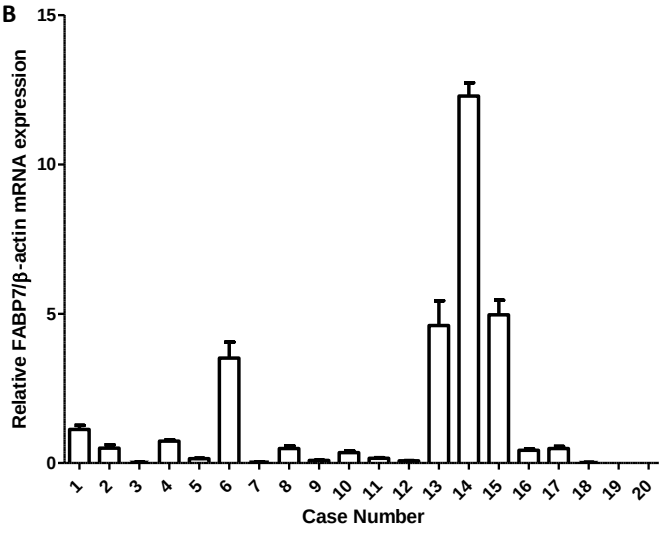
The FABP7 antibody (clone G13) with the availability of a blocking peptide was used validate the specificity of the staining. Top Left: x20 A FABP7 positive TN IDC breast cancer case stained for using the R&D FABP7 antibody. Top Right: x20 The same IDC TN breast cancer case stained with the G13 Santa Cruz FABP7 antibody. Bottom: x20 The addition of the FABP7 blocking peptide; staining was eliminated.

3.4.10 FABP7 mRNA in patient samples is correlated with IHC protein expression

RNA was extracted from breast cancer tissue curls and the corresponding slides were stained for FABP7 with IHC. Figure 3.14 shows the FABP7 IHC Allred scores and FABP7 qPCR results from FFPE tissue and the correlation of FABP7 protein and mRNA expression in FFPE patient samples. Case 14 had the highest amount of FABP7 mRNA and an Allred score of 7; cases 19 and 20 had barely detectable levels of FABP7 mRNA and Allred scores of 0 and 2 respectively. The expression of FABP7 mRNA and the Allred score were significantly associated with a p value of 0.0004 with Spearman's correlation.

A

Case Number	Proportion	Intensity	Total Score
1	0	0	0
2	2	2	4
3	0	0	0
4	2	1	3
5	0	0	0
6	1	1	2
7	0	0	0
8	1	1	2
9	0	0	0
10	2	2	4
11	0	0	0
12	0	0	0
13	4	2	6
14	4	3	7
15	5	3	8
16	3	4	5
17	1	1	2
18	0	0	0
19	0	0	0
20	1	1	2



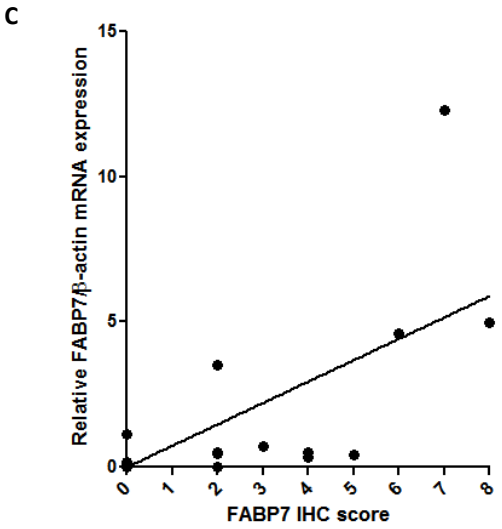


Figure 3.14 FABP7 mRNA and protein expression in patient samples

RNA was extracted from the 20 available tissue curls and the corresponding sections were stained using IHC for FABP7. The Allred scores are in A; the strongest staining case was 15. The relative FABP7 mRNA expression is in B, case 15 had the second highest expression, case 14 the highest. C) The correlation of the FABP7 scores and the relative FABP7 mRNA expression. The FABP7 mRNA results and FABP7 IHC scores were significantly correlated (p=0.0004, Spearman's correlation).

3.4.11 FABP7 expression and clinicopathological features

Ethnicity, lymph node involvement, grade (there were no grade 1 cases) and lymphovascular invasion were not associated with FABP7 expression. Stage was associated with FABP7 expression; cases that were FABP7 positive tended to be of a lower stage (p=0.039). Both basal cytokeratins CK14 and CK5/6 positivity were associated with FABP7 positivity (p=0.048 and p=0.031 respectively) (figure 3.15) (table 3.2).

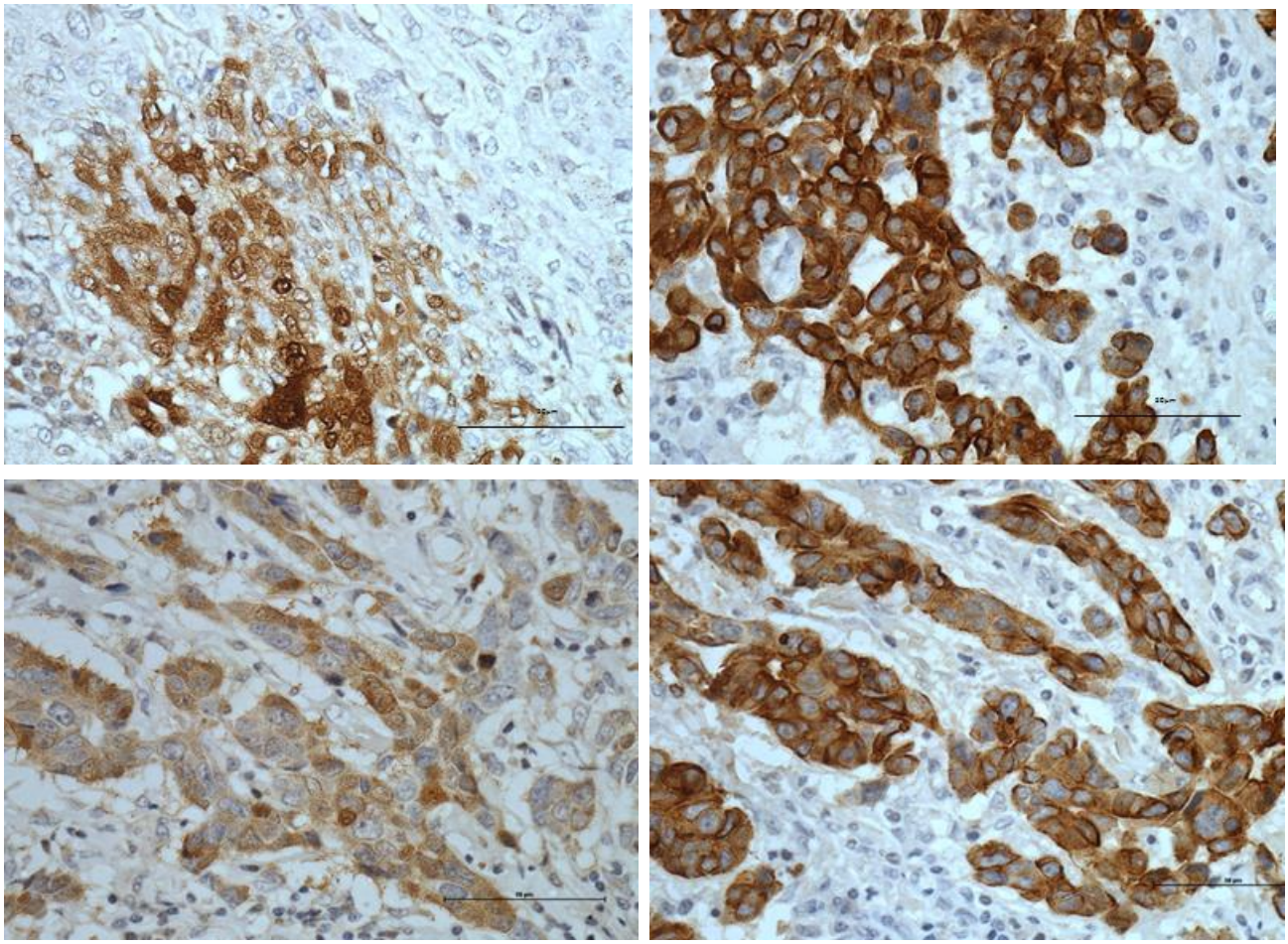


Figure 3.15 FABP7 positivity is associated with CK5/6 and CK14 positivity

Top Left: x40 A FABP7 positive IDC breast cancer case; Top Right: x40 The same breast cancer case stained positively with CK5/6. Bottom Left: x40 A different FABP7 positive IDC case; Bottom Right: x40 The same case stained positively for CK14.

Table 3.2 Factors associated with FABP7 expression

		FABP7 Score							
	Overall	***0***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***P for X^2 test***
No. of patients	99									
Ethnicity, *N (%)*										0.939
Chinese	59 (60.6)	12 (60)	1 (100)	4 (100)	7 (63.4)	7 (53.8)	4 (44.4)	7 (63.6)	17 (56.7)	
Malay	21 (21.2)	4 (20)	0	0	1 (9.1)	5 (34.5)	1 (11.1)	2 (18.2)	8 (26.7)	
Indian	15 (15.2)	3 (15)	0	0	2 (12.2)	1 (7.7)	3 (33.3)	2 (18.2)	4 (13.3)	
Others	4 (3.0)	1 (5)	0	0	1 (9.1)	0	1 (11.1)	0	1 (3.3)	
Lymph node involved, *N (%)*										0.612
Yes	41 (42.3)	12 (60)	0	2 (50)	4 (36.4)	6 (50)	3 (33.3)	3 (27.3)	11 (37.9)	
No	56 (57.7)	8 (40)	1 (100)	2 (50)	7 (63.4)	6 (50)	6 (66.7)	8 (72.7)	18 (62.1)	
Unknown	2									
Stage, N,(%)										0.039[a]
Early (Stage 1-2)	66(66.7)	11 (55)	1 (100)	3 (75)	6 (54.5)	5 (38.5)	9 (100)	10 (90.9)	21 (70)	
Late (Stage 3-4)	33(33.3)	9 (45)	0	1 (25)	5 (45.5)	8 (61.5)	0	1 (9.1)	9 (30)	
Grade [d], ***N (%)***										0.382
Grade 2	17 (17.7)	4 (20)	1 (100)	1 (25)	2 (18.2)	2 (15.4)	0	1 (9.1)	6 (21.4)	
Grade 3	79 (82.3)	16 (80)	0	3 (75)	9 (81.8)	11 (84.6)	8 (100)	10 (90.9)	22 (78.6)	
Unknown	3									
Lymphovascular invasion, *N (%)*										0.686
Present	33 (37.5)	8 (44.4)	1 (100)	2 (66.7)	3 (30)	5 (41.7)	3 (42.9)	4 (15.4)	7 (21.4)	
Absent	55 (62.5)	10 (55.6)	0	1 (33.3)	7 (70)	7 (58.3)	4 (57.1)	7 (84.6)	19 (78.6)	
Unknown	11									
CK14 status, *N (%)*										0.048[a]
Negative	41 (54%)	17 (85)	0	3 (75)	5 (45.5)	6 (50)	2 (22.2)	5 (45.5)	16 (59.3)	
Positive	54 (46%)	3 (15)	1 (100)	1 (25)	6 (54.5)	6 (50)	7 (77.8)	6 (54.5)	11 (40.7)	
Unknown	4									

Table 3.2 continued

CK 5/6 status, *N (%)*										0.031[a]
Negative	24 (29.1)	8 (47.1)	2 (66.7)	3 (100)	1 (9.1)	2 (16.7)	1 (11.1)	3 (30)	6	
Positive	63 (70.9)	9 (52.9)	1 (33.3)	0	10 (90.9)	10 (83.3)	8 (88.9)	7 (70)	18	
Unknown	12									
CK14 and CK5/6 status, N *(%)*										0.008[a]
Negative	55 (65.5)	16 (94.1)	0	3 (100)	6 (54.5)	6 (54.5)	2 (22.2)	6 (60)	16 (72.7)	
Postive	29 (34.5)	1 (5.9)	1 (100)	0	5 (45.5)	5 (45.5)	7 (77.8)	4 (40)	6 (27.3)	
Unknown	15									
CK14 or CK5/6 status, N *(%)*										0.061
Negative	36 (36.7)	10 (50)	0	3 (75)	1 (9.1)	3 (23.1)	1 (11.1)	4 (36.4)	14 (48.3)	
Positive	62 (63.3)	10 (50)	1 (100)	1 (25)	10 (90.9)	10 (76.9)	8 (88.9)	7 (63.6)	15 (51.7)	
Unknown	1									

a) Statistically significant d) there were no cases that were grade 1.

3.4.12 FABP7 expression is not related to tumour size or patient age

A Mann-Whitney U test was carried out to look for significance between FABP7 expression and tumour size and patient age. The median age for FABP7 negative cases (those with a score of 6 or less) was 52; the median age for FABP7 positive cases (a score of 7 or 8) was 55; this difference was not statistically significant (p=0.724) (table 3.3). The median tumour sizes were 3.5 and 3.3cm for FABP7 negative and positive cases respectively (p=0.236) (table 3.3).

	Overall	FABP7 negative	FABP7 positive	P for X2 test
Age (median, years)	53	52	55	0.724
Tumor size (median, cm)	3	3.5	3	0.236

Table 3.3 The association of FABP7 expression and patient age and tumour size

This table shows the association of FABP7 expression with patient age and tumour size. The statistical software used for this analysis automatically converted from chi-squared test to Fisher's exact test for numbers lower than five.

3.4.13 FABP7 expression is associated with FAS expression

It was hypothesised that FABP7 expression is associated with FAS expression due to the involvement of FAS in the production of FAs. FAS and FABP7 were stained using IHC and assessed using a modified Allred score; FAS and FABP7 score cross tabulation is in table 3.4. FABP7 was positively associated with FAS expression (p=0.004) (photomicrograph figure 3.16)

		FAS score								
		0	2	3	4	5	6	7	8	Total
FABP7	0	7	0	1	0	5	3	1	3	20
score	2	0	1	0	0	0	0	0	0	1
	3	1	0	0	1	0	1	1	0	4
	4	1	0	0	2	2	2	2	2	11
	5	2	0	1	2	2	2	1	1	11
	6	0	0	2	2	2	0	0	3	9
	7	2	0	1	1	3	1	0	1	9
	8	6	1	1	2	4	2	7	6	29
Total		19	2	6	10	18	11	12	16	94

Table 3.4 FABP7 and FAS staining are positively associated

This table shows the cross-tabulation of FABP7 scores with FAS scores. FABP7 expression was positively associated with FAS expression (p=0.004)

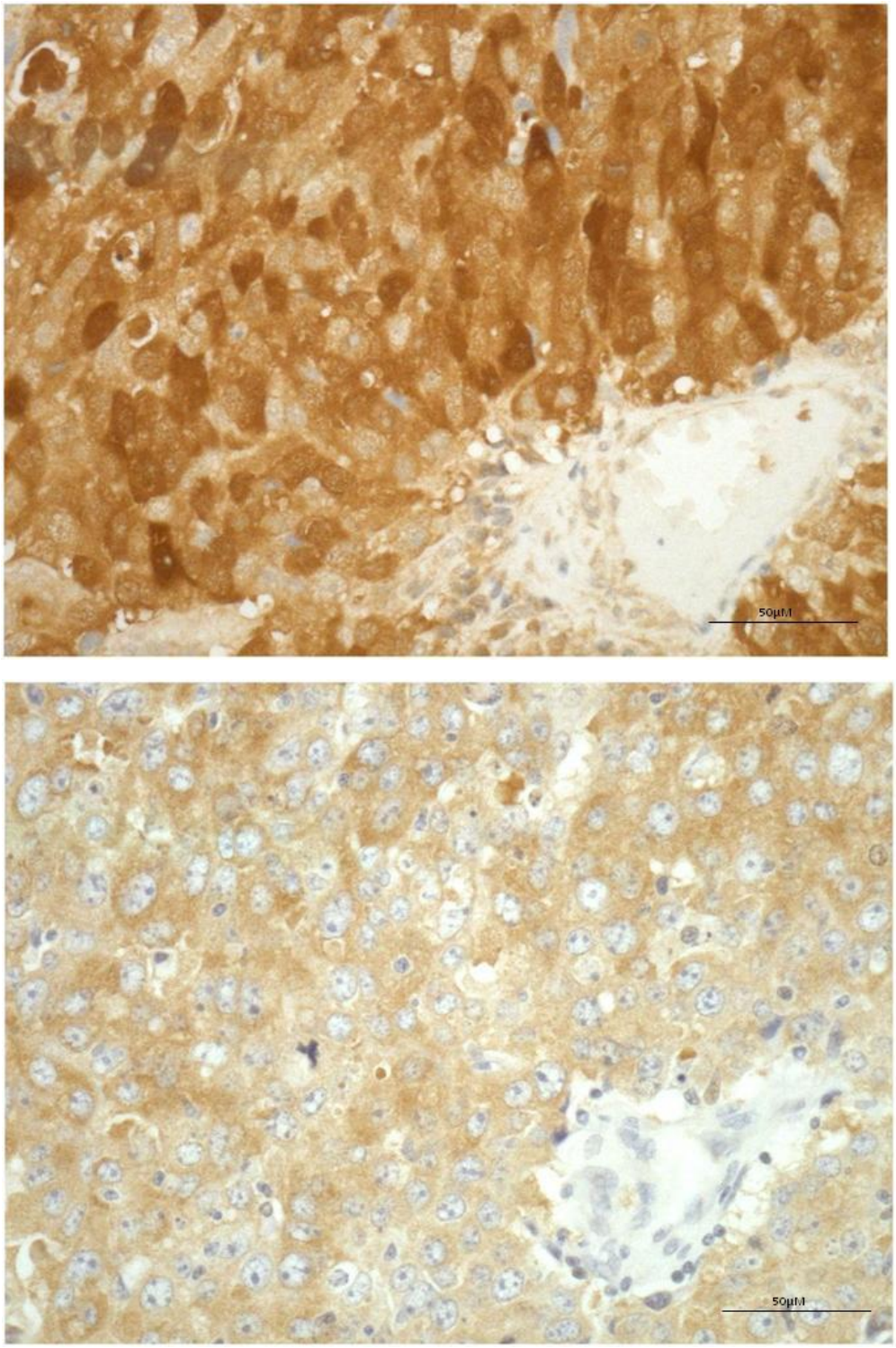
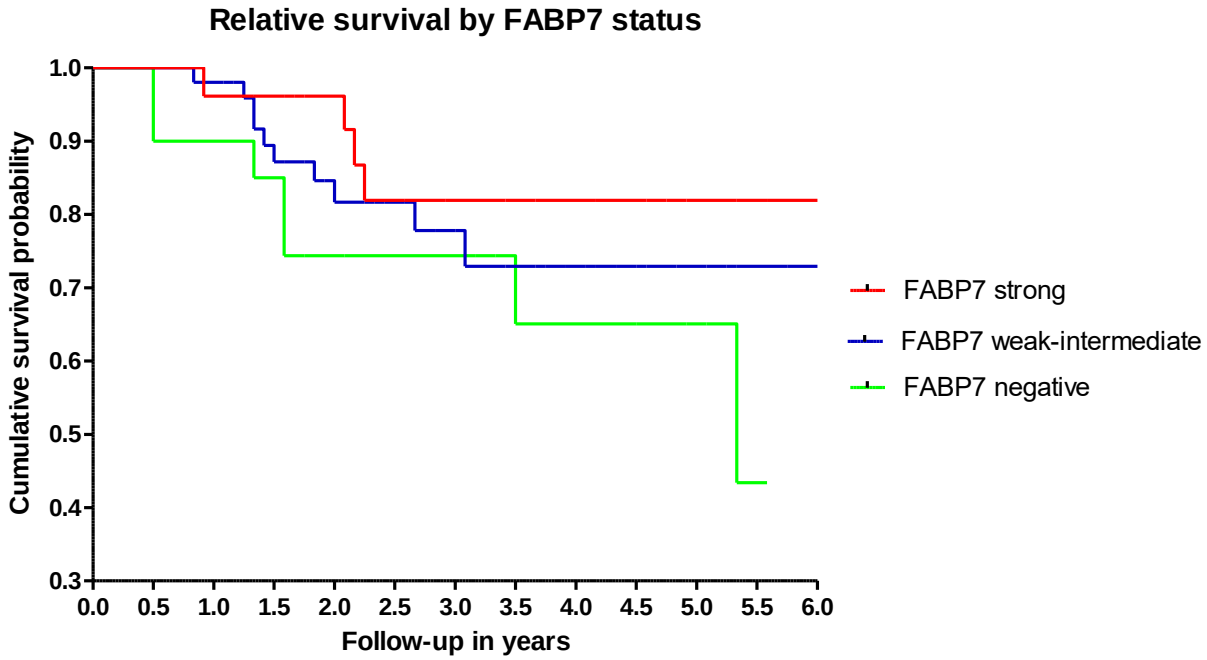


Figure 3.16 The association of FABP7 and FAS

IHC staining of a TN breast IDC case. Top: x40 Strong positive FABP7 staining localised to both the nucleus and cytoplasm of cells. Bottom: x40 Positive FAS staining in the same IDC case; FAS staining was localised to the cytoplasm of cells.

3.4.14 FABP7 expression is associated with improved patient survival

FABP7 staining was grouped into cases that were negative, weak to intermediate (scores 2 to 6) and strong (scores 7 and 8). A Kaplan-Meier survival graph and analysis were carried out. The graph in figure 3.17 shows that cases with strong FABP7 expression have a better survival than cases that were weak-intermediate and negative; the FABP7 negative cases had the poorest survival. The 3 year relative survival rate (RSR) for FABP7 negative cases was 74.4% (95%CI 55-93.8%); for intermediate-weak cases the RSR was 72.9% (95%CI 57.4-88.5%) and for FABP7 positive cases it was 81.9% (95%CI 65.8-98.1%) However the relationship between FABP7 and survival was not significant (p=0.291).



	Year(s) following diagnosis with breast cancer					
	0	1	2	3	4	5
FABP7 negative						
Number of patients entering interval	19	18	17	14	7	2
Relative Survival %	100.0	90.0	85.0	74.4	65.1	43.4
(95%CI)		76.6-103.1	69.4-100.6	55.0-93.8	41.0-89.1	51.1-81.6
FABP7 weak intermediate						
Number of patients entering interval	50	45	28	15		
Relative Survival %	100.0	95.9	81.7	72.9		
		90.3-101.5	70.1-93.3	57.4-88.5		
FABP7 positive						
Number of patients entering interval	25	25	20	17		
Relative Survival %	100.0	96.2	91.6	81.9		
(95%CI)		88.8-103.5	80.3-102.8	65.8-98.1		

Figure 3.17 FABP7 and patient survival

FABP7 IHC expression was grouped into negative, weak-intermediate and strong staining and then a Kaplan Meier survival analysis was carried out. Results show that strong positive FABP7 had the best overall survival and FABP7 absent cases the poorest relative survival.

3.5 Discussion

3.5.1 Western blot optimisation

This chapter addresses the importance of optimising a western blot protocol in order to successfully detect a protein of interest, in this case FABP7. If it were not for the evidence illustrating that FABP7 is present in human breast cancer tissues, the assumption could have been made from the original western blots that FABP7 protein is not present in breast cancer cell lines thus impacting future work. There are many variables that have been taken in to account and optimised.

The first and most critical step in producing samples for SDS PAGE analysis and subsequent Western blotting is the selection of a suitable lysis buffer. This should always be a considered decision, assessing the benefits of each buffer and the nature of the protein of interest. Only through careful consideration can we ensure that proteins are effectively released but also solubilised. Once the lysis step is fully optimised several other stages need to be carefully assessed. The results showed for FABP7 that there was negligible difference in the intensity of bands generated from the lysis buffer and RIPA buffer; however the sample buffer lysis protocol resulted in a much stronger band for the same amount of total protein loaded. One reason could be that sample buffer lysis protocol results in a higher yield of FABP7 protein from all cellular fractions.

The choice of antibody should be made ideally based on quality, which can be determined by reviewing reference sources. However in many instances the choice of commercially available antibodies can be limited and in such circumstances comparison between competitors should be considered to ensure selection of the best antibody for the task. One should consider the differences between polyclonal and monoclonal antibodies and any advantages they confer. For example monoclonal antibodies recognise only one epitope and consequently produce lower background. However polyclonal antibodies recognise multiple epitopes within the protein of

interest which means although they may produce more noise they may also facilitate better detection of low abundant or difficult to detect proteins. In this study both of the antibodies employed were polyclonal and we illustrated that the R&D antibody proved to be superior to the SC anti-FABP7 primary antibody which produced more noise and therefore poorer band detection. This could have in part been explained by the difference in clonality between the antibodies. However it is also worthwhile noting that the availability of the blocking antigen is also an extremely useful tool in ensuring that any results produced are accurate and specific, hence our inclusion of the data produced using the Santa Cruz antibody. This becomes increasingly important in situations where multiple bands are detected within a given sample to ensure that the correct band or bands of interest are selected for analysis. In our study although only one band was detected in each sample the blocking peptide provided reassurance of specificity which was important due to the difficulties experienced with detecting the protein in samples of interest. The use of the SC antibody with the corresponding blocking peptide enabled us to determine whether the bands detected were indeed specific. The bands were eliminated with the addition of the blocking peptide indicating that the antibody is specific and the bands detected are FABP7 protein.

Other points to consider include the choice of blocking reagent. In our study this proved to be essential since BSA blocking led to a decreased signal-to-noise ratio, making visualisation of any bands difficult. It is also important to ensure that the blocking reagent does not adversely affect the linear dynamic range where absolute quantification is required. The selection of reducing versus non-reducing conditions must also be considered carefully in order to ensure that antibody epitopes are present and correct thereby ensuring optimal detection. The results showed that reducing conditions were optimal since the threshold of detection was unchanged by the presence of the reducing agent and the migration profile was more favourable.

The final consideration should be given to the percentage and type of gel utilised. Selection of the correct percentage of acrylamide is essential in obtaining the best migration profile and subsequently the highest quality Western blot. Proteins of interest must be considered alongside any proteins used as a loading control to ensure that the gel selected is complimentary to both and in many situations a gradient gel provides the ideal solution. It is also worth considering the benefits of consistency and reproducibility provided by commercially available pre-cast gels. These results demonstrated that the use of gradient gels facilitated the detection of bands that were much stronger overall than the 12% in-house gel, most likely the result of improved protein migration and separation.

3.5.2 FABP7 expression in breast cancer cell lines

This is the first study to detect FABP7 protein in a panel of commonly used breast cancer cell lines; without the optimisation of each step it would not have been possible. This probably accounts for the paucity of studies using breast cell lines for investigating FABP7 and those that have, have transfected the protein into the cell lines. BT-20 cells expressed the most protein followed by MDA-MB-231 and T47D cells that had comparable protein expression. MCF-10a and HS578T cells had the lowest amount of FABP7 protein as assessed by band densitometry. BT-20 and MDA-MB-231 cells are TN and of basal-like phenotype so it is logical to expect that these cells would have the highest expression considering the association of FABP7 with BLBC. As would be expected BT-20 cells also expressed the most FABP7 mRNA however FABP7 mRNA levels in MDA-MB-231 cells were considerably lower. Protein and mRNA do not necessarily correlate and it is possible that there is a high turnover of FABP7 mRNA in MDA-MB-231 cells or that there is more RNA degradation in these cells. The same may be true of the reverse; SKBR3 cells have comparably high FABP7 mRNA expression but lower protein expression, FABP7 protein degradation could be a factor. Overall FABP7 protein and mRNA expression is low in the breast cell lines, particularly

compared to expression in U251 cells and it is therefore debatable whether knock-down experiments would notably alter FABP7 protein or mRNA expression in breast cell lines. There is ample evidence, including the present study, documenting FABP7 expression in TNBC particularly in patient samples (Zhang *et al.,* 2010; Tang *et al.*, 2010). There are several putative reasons for the low abundance of FABP7 in cell lines; one is that there is an absence of biological factors that would be in the tumour microenvironment *in vivo* that are vital for expression to occur. For instance a 3D environment may be needed. Studies have shown that surrounding stromal tissue is important when considering gene expression and tumour development and progression; a limitation is that the cell-line models in the present study do not have such composition and perhaps this why expression levels of FABP7 differ from those reported in patient samples (Finak *et al.*, 2008). Other factors to consider are vascularisation, hypoxia and macromolecules such as fatty acids that a tumour may or may not be exposed to *in vivo* compared to *in vitro* cell lines. However despite such limitations it is still necessary to quantify FABP7 in breast cancer cells lines in order to choose an appropriate model for further investigation.

3.5.3 FABP7 mRNA and protein correlation in patient samples

To further validate the antibody used in the present study and understand FABP7 expression RNA was extracted from 20 FFPE patient samples and FABP7 mRNA quantified with qPCR. The qPCR results were compared to the IHC expression assessed with a modified Allred score. There was a significant association between the FABP7 IHC score and mRNA expression. This helps to demonstrate that the antibody used in IHC is sensitive and specific, as high mRNA levels do equate to high protein expression. It is useful to know that FABP7 mRNA and protein are associated as it helps provide possible explanations as to why protein expression in the breast cell lines is low; it is likely that there are factors preventing FABP7 mRNA expression or its stability in the breast cancer cell lines rather than an issue with mRNA to protein translation or protein

stability. Ascertaining the mechanisms involved in FABP7 mRNA expression would be beneficial in understanding why FABP7 is in low abundance in cell lines and allow appropriate cell line models to be selected for further investigation of FABP7.

3.5.4 FABP7 is expressed in patient samples

To further investigate the importance of FABP7 in breast cancer it was decided to look at expression levels in a cohort of Malaysian TN breast cancer cases to see if lFABP7 expression patterns followed those in western cohort studies (Zhang *et al.*, 2010; Tang *et al.*, 2010; Alshareeda *et al.*, 2012). FABP7 expression was then compared to clinicopathological features. FABP7 staining was predominantly cytoplasmic with some nuclear staining also present in positive cases; this staining pattern is in agreement with other studies (Zhang *et al.*, 2010; Tang *et al.*, 2010; Alshareeda *et al.*, 2012). In the present study around 80% of cases had some level of FABP7 staining; using a score of 7 or 8 over 40% of cases were strongly positive. This is higher than the reported percentage positivity of 15.6% in an unselected breast cancer cohort (Zhang *et al.*, 2010). When looking BLBC within their cohort 38.4% were FABP7 positive; this is comparable to the present study (Zhang *et al.*, 2010). This could be a reflection of the majority of cases being of basal-like phenotype in the present study. The staining in patient samples was validated by use of a blocking peptide; staining was eliminated indicating specificity for FABP7. The presence of FABP7 in patient samples further confirms its relevance in breast cancer particularly in those of TN phenotype.

3.5.5 FABP7 is associated with tumour stage and basal-like phenotype.

FABP7 IHC scores were compared to patient features such as stage, patient age and grade and to the staining of CK5/6 and CK14. Interestingly FABP7 positivity was associated with a lower stage;

this could indicate that its presence is of favourable prognosis and is therefore consistent with the work by Zhang *et al* (2010). There were no grade 1 cases in the present study so a relationship between FABP7 and grade would not have been possible to elucidate, although Zhang's cohort was unselected, larger and consisted of grade 1 tumours, a relationship of grade with FABP7 was not present. This is in contrast to Tang *et al.* (2010) who found FABP7 positive breast cancer cases were more likely to be of a higher grade, as did another study looking at FABP7 in astrocytomas; nuclear FABP7 positivity was associated with higher grade. Ethnicity and patient age were not factors associated with FABP7 status. Neither was lymph-node involvement; this is contrast to findings showing that lymph-node involvement was lower in FABP7 positive cases (Zhang *et al.*, 2010). Reasons for the difference in significant findings could be due to the size of the cohort or the use of cut of points and staining interpretation. As expected, FABP7 was significantly associated with CK5/6 and CK14 expression; this in conjunction with other molecular and immunohistochemical studies demonstrating the relationship between FABP7 and BLBC (Zhang *et al.*, 2010; Tang *et al.*, 2010).

3.5.6 FABP7 and FAS expression are significantly associated

FAS is responsible for the conversion of malonyl CoA to palmitate which can then be altered to synthesize other FAs. It was hypothesised that high expression of FAS may confer positivity of FABP7 to enable the transportation and solubilisation of increased palmitate and FA production. The present study found that FABP7 scores were significantly positively correlated with FAS IHC scores. In contrast one study used IHC to show that liver FABP was commonly over-expressed in gastric cancers however its over-expression was not associated with over-expression FAS (Hashimoto *et al.*, 2004). Cell line models are required to explore the relationship further and to ascertain whether the expression of the markers are directly related or which marker is responsible for the over-expression of the other and if palmitate is involved.

3.5.7 FABP7 expression is associated with increased survival

Cases were grouped into those that were completely negative for FABP7, weak to intermediate (scores 2-6) and strong positive cases (scores 7&8); the data were then analysed with Kaplan Meier survival analysis. Although not significant there was a trend that strong positive cases had the best overall survival probability followed by weak-intermediate FABP7 cases and completely negative cases having the poorest survival probability. A larger cohort and longer follow-up time period may be beneficial in further understanding the relationship between FABP7 and survival. The findings of the present study are in conjunction with results from Zhang *et al*. (2010) though in contrast to another study using a breast cancer cohort and studies looking at glioma and melanoma where FABP7 positivity conferred poorer survival (Kaloshi *et al.*, 2007; Tang *et al.*, 2010; Goto *et al.*, 2010). The increased survival probability with FABP7 expression in the present study ties in with the association of FABP7 with lower stage. A limitation of the study by Tang *et al*. (2010) was that there were only a small number of TN cases on the cohort; the association of FABP7 with poor survival could be a reflection of TN cancers having poor survival rates compared to other breast cancer subtypes. A recent study looked at the cellular localisation of FABP7; nuclear localisation was associated with high tumour grade and stage and interestingly in BLBCs nuclear FABP7 had improved prognosis and less chance of reoccurrence than cases with cytoplasmic staining alone (Alshareeda *et al.*, 2012). Upon observation the majority of positive cases in the present study had a combination of nuclear and cytoplasmic FABP7 localisation; nuclear staining alone was not observed by itself perhaps providing a reason as to why significant improved prognosis was seen in this TN/BLBC cohort. This is in contrast to a study on FABP7 in gliomas; nuclear FABP7 was associated with EGFR expression and in turn this was an indicator of poor prognosis (Liang *et al.*, 2006). It is possible that the relative abundance of FA and the localisation of FABP7 within cells influence tumour phenotype; as suggested by Mita *et al.* (2010) the relative abundance of AA and DHA can influence which pathways are activated. For example

FABP7 is hypothesised to shuttle DHA to the nucleus to activate PPAR-γ and result in down regulation of pro-migratory genes or it shuttles AA to cytoplasmic pathways such as COX2 to initiate pro-migratory pathway activation (Mita *et al.*, 2010). It is possible that there are other pathways in breast cancer that are activated and perhaps different FAs are present thus explaining the different associations of nuclear and cytoplasmic FABP7 and how it may influence tumour phenotype and patient outcome. This evidence suggests that cellular localisation of FABP7 and interaction with FAs are important in patient survival and tumour progression and thus FABP7 expression warrants further investigation.

Very little FABP7 mRNA and protein was detected in any of the breast cancer cell lines tested though FABP7 mRNA and protein was present in 20 samples selected for mRNA analysis. In addition the majority of patient samples expressed FABP7 at the protein level detected by IHC. One reason that FABP7 is in low abundance in the cells lines tested could be that FABP7 is hypermethylated or hypoacetylated or that a factor is missing *in vitro* that is required for FABP7 expression, such as certain FAs.

3.6 Conclusions

FABP7 protein and mRNA can be detected in breast cell lines; BT-20 breast cell lines expressed the highest amount of both FABP7 protein and mRNA of the cell lines tested. FABP7 expression in patient samples is associated with lower stage and the basal-like phenotype as assessed by cytokeratins 5/6 and 14 expression. FABP7 expression is associated with improved patient survival though the trend was not significant.

4 What regulates FABP7 expression in triple negative breast cancer cell lines?

4.1 Introduction

Evidence suggests the mRNA expression of various FABPs is altered by methylation and acetylation of the DNA. For example differences in FABP7 methylation was observed between subsets of chronic lymphocytic leukaemia (CLL); FABP7 was listed as a methylated gene in the immunoglobulin heavy-chain variable mutated genes type of CLL (Kanduri *et al.*, 2010). FABP7 has been investigated as one of several genes that were hypomethylated in glioblastomas compared to the normal brain; this results in over-expression of FABP7 in glioblastomas (Etcheverry *et al.*, 2010). FABP3, also known as *MDGI*, has been found to be similar to FABP7 with respect its effects in breast cancer cell lines. A study by Huynh *et al*. (1996) demonstrated that *FABP3* is hypermethylated and thus silenced in both breast cancer cell lines and a selection of patient breast tumours. It is postulated that FABP7 may be hypermethylated in a similar fashion in breast cancers. Currently there is little information regarding changes in the acetylation status of FABPs in cancer. However it has been reported that the expression of FABPs can be altered through changes in histone acetylation. Trichostatin A (TSA), an HDAC inhibitor, was demonstrated to increase FABP4 expression in RAW 264.7 macrophages (Coleman *et al.*, 2010). Treatment with TSA was also enough to negate the inhibitory effect of linoleic acid on FABP4 expression (Coleman *et al.*, 2010). Drugs, such as *5-Aza-2*'-deoxycytidine (AZA), a DNA demethylating agent, and TSA, can be used to investigate global changes in epigenetic states and moreover used to explore whether FABP7 in TN breast cancer cell lines is regulated by epigenetic mechanisms.

Fatty acids (FA) can alter gene expression (section 1.6.2) through direct or indirect mechanisms or transcriptional or posttranscriptional action. In particular they alter the expression or FABPs. It was found that FABP4 levels could be altered in RAW264.7 macrophages by exposing the cells to FAs (Coleman *et al.*, 2010). Unsaturated FAs such as linolenic acid and palmitoleic acid decreased FABP4 mRNA and protein; however the unsaturated FA eicosapentaenoic acid significantly decreased FABP4 protein but mRNA levels were not different from the control (Coleman *et al.*, 2010). FABP3 and FABP5 mRNA and protein have been found to be increased in rat pancreatic islet cells and NS-1E cells (a rat pancreatic β-cell line) by treatment with palmitate and oleate (Hyder *et al.*, 2010). FABP1, also known as Liver-FABP, can be up-regulated at both the mRNA and protein level by oleate in rat hepatoma cells (Meunier-Durmort *et al.*, 1996). Other long chain FAs that increased FABP1 expression in rat hepatoma cells included palmitate, arachidonic acid (AA) and linoleic acid. FAs can work in conjunction with other molecules to alter expression; docosahexaenoic acid (DHA) activates the retinoid X receptor (RXR) by acting as a transcription factor when bound to FABP7; however this causes the activation of RXR rather than FABP7 itself but it is plausible that there may be a feedback mechanism involved that in turn alters the expression of FABP7 (Liu *et al.*, 2012). FABP3 has been shown to have similarities to FABP7, particularly in regards to the effects of FABP3 expression in breast tissue; FAs including palmitate, oleate and AA increased FABP3 mRNA at least two fold (Chang *et al.*, 2001). The relationship between FABP gene expression and FA is complex. FABP7 binds to palmitate, oleate, DHA and AA and evidence suggests that these FAs alter FABP gene expression; MDA-MB-231 and BT-20 cells were exposed to FAs in order to investigate changes in FABP7 expression.

4.2 Aims and hypothesis

4.2.1 Aims

i) To investigate whether FABP7 expression is changed by AZA and TSA in TN breast cancer cell lines.

ii) To investigate whether the FAs, oleate, palmitate, AA and DHA alter expression of FABP7 in TN breast cancer cell lines.

iii) To investigate whether any of the compounds have synergistic or combined effects on FABP7 expression when used together.

4.2.2 Hypothesis

i) FABP7 expression in TN breast cancer cell lines is affected by methylation and acetylation and the availability of FAs.

4.3 Materials and Methods

4.3.1 Cell culture and dosing experiments

Cells were seeded at a density of $0.8x10^6$ per T25 flask. For the AZA, TSA and FA experiments they were treated as described in section 2.3. The final doses for the FAs were chosen as follows palmitate 50µM, oleate 100µM, AA 10µM and DHA 10µM; these doses were chosen based on dose response results (section 1.10). The doses of AZA and TSA were chosen based on results from collaborators at the University of Bristol who were looking at changes in gene expression in the same breast cancer cell lines. In addition to this BT-20 cells were treated with AZA and TSA combined; cells were exposed to 2.5µM AZA for 48 hours when 0.5µM TSA was added, the cells were then harvested after 24 hours TSA exposure and therefore 72 hours of AZA exposure. To test the combined effects of AZA and FAs BT-20 cells were exposed to 2.5µM AZA for 48 hours; after this time the growth medium was changed to contain either one of the FAs, at this time a second dose of 2.5µM AZA was also given. AZA alone and fatty acid alone controls were set up for this experiment.

4.3.2 RNA extraction and PCR

For these experiments cells were subjected to RNA extraction using the Bioline kit (section 2.6.1). RNA was converted to cDNA (section 2.7.1) and then FABP7 and ER mRNA was quantified using qPCR (section 2.7.4). B-actin was used as a reference gene.

4.3.3 Protein extraction and western blotting

The combinations of AZA and FA treatment that gave the largest increase in FABP7 mRNA were repeated to extract protein for western blotting. This time point was not increased to allow for protein production because preliminary work at increased time points showed cell death

increased and FABP7 protein levels were unchanged. Protein extraction took place with Laemmli x2 sample buffer (section 3.4.4); the lysates were then subjected to western blotting using a gradient gel (section 3.3.5).

4.3.4 Statistical analysis

Analysis was carried out on the results of 3 independent experiments that had been carried out in triplicate unless otherwise stated. The statistical package Graphpad Prism 5 was used to produce graphs and produce statistics for these experiments. Whenever there was more than one comparison against the control a one-way ANOVA with a Bonferroni post-hoc test was used to highlight differences; a students paired t-test was then carried out to confirm significant differences. Data are presented as mean ± standard error of the mean (SEM).

4.4 Results

4.4.1 AZA and TSA increase ER mRNA expression in BT-20 and MDA-MB-231 cells

Expression of oestrogen receptor (ER) mRNA was measured (from the same RNA extractions used to measure FABP7 expression) after treatment with 0.5μM TSA for 24 hours and 2.5μM AZA for 72 hours. This was as a control to demonstrate that the AZA and TSA were causing changes in gene expression; ER in the same breast cancer cell lines has been found to be re-expressed after treatment with these drugs. The results are in figure 4.1; TSA increased ER mRNA by 6.1 fold (A: MDA-MB-231 cells p=0.0025) and 8.1 fold (B: BT-20 cells p=0.0001). AZA also significantly increased ER mRNA in both cell lines, 32.4 fold (A: MDA-MB-231 cells p=0.0001) and 33.5 fold (B: BT-20 cells p=0.0001).

4.4.2 AZA but not TSA increases FABP7 mRNA in MDA-MB-231 cells

MDA-MB-231 cells were first treated with 1μM AZA for up to 72 hours or 0.25μM or 0.5 μM TSA for 24 hours. As shown in figure 4.2, FABP7 mRNA was increased after 48 (C) and 72 (D) hours by 1.53 and 1.45 fold respectively however the increase was only statistically significant after 48 hours (p=0.0311). There were no significant changes in FABP7 mRNA due to treatment with TSA (A) or AZA for 24 hours (B). Next a higher dose of AZA was used to treat the MDA-MB-231 cells and FABP7 expression measured after 48 and 72 hours as no increase was noted at 24 hours. Although this time 1μM AZA resulted in a 1.53 fold increase in FABP7 mRNA, it was not statistically significant. As in figure 4.3 a 2.5μM dose of AZA gave a significant increase at both 48 (A) and 72 (B) hours, 1.56 (p=0.0073) and 2.11 (p=0.0167) respectively. There was not any observable change in cell death as a result of AZA or TSA treatment.

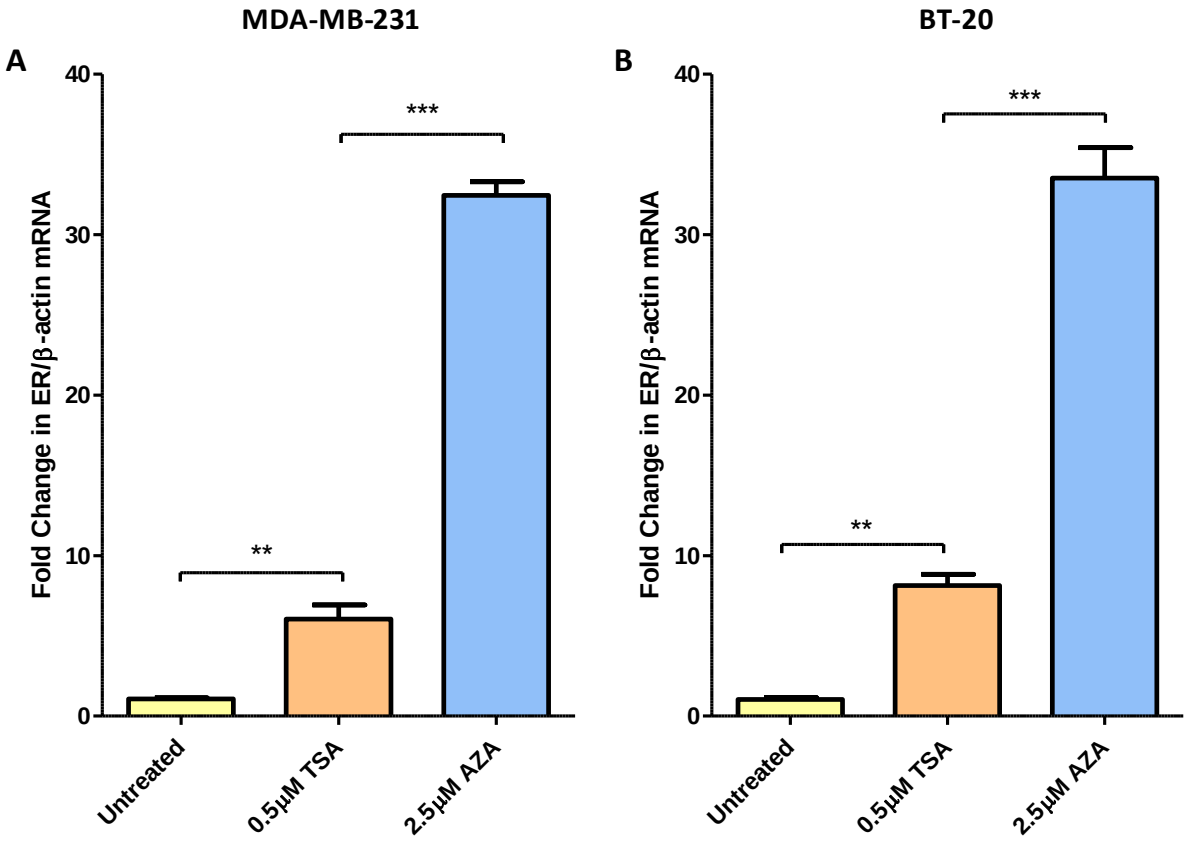


Figure 4.1 Fold change in ER mRNA after treatment with TSA or AZA

MDA-MB-231 and BT-20 cells were treated with 0.5μM TSA for 24 hours and 2.5μM AZA for 72 hours. Results are expressed as fold change relative to ER mRNA in untreated cells in SFM alone. A) Fold change in ER mRNA in MDA-MB-231 cells: TSA increased ER mRNA 6.1 fold** (p=0.0025); AZA increased ER mRNA 32.4 fold*** (p=0.0001). B) Fold change in ER mRNA in BT-20 cells: TSA increased ER mRNA 8.1 fold*** (p=0.0001); AZA increased ER mRNA 33.5 fold*** (p=0.0001). Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

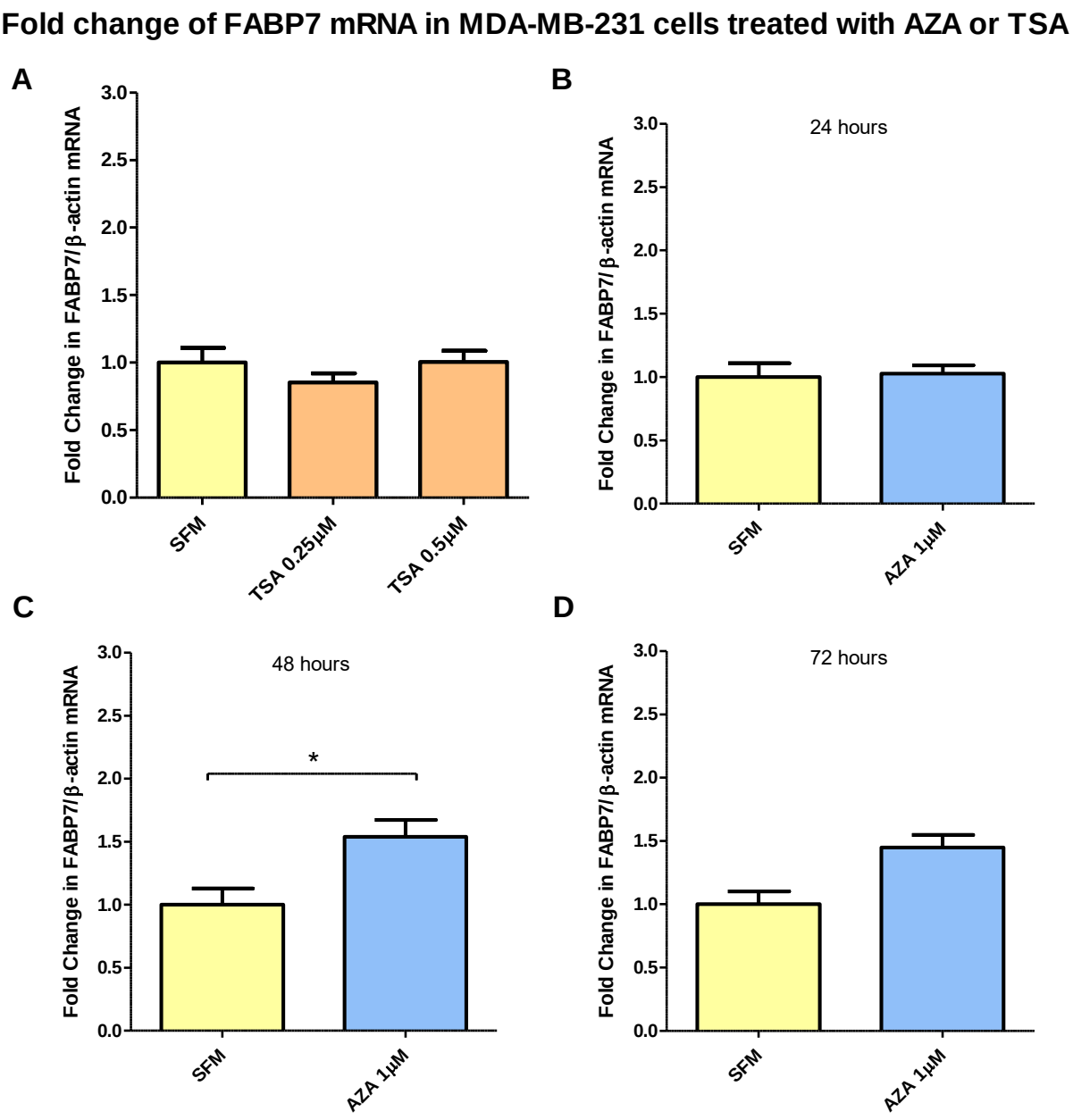


Figure 4.2 Fold change of FABP7 mRNA in MDA-MB-231 cells treated with AZA or TSA

Results are expressed as fold change relative to FABP7 mRNA in cells in SFM alone. A) Fold change in FABP7 mRNA after 0.25µM (0.81 fold change) and 0.5µM (1.00 fold change) TSA treatment for 24 hours. B) Fold change in FABP7 mRNA after 24 hours 1µM AZA treatment (1.02 fold change). C) Fold change in FABP7 mRNA after 48 hours 1µM AZA treatment *(1.53 fold change p=0.0311). D) Fold change in FABP7 mRNA after 72 hours 1µM AZA treatment (1.44 fold change) Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

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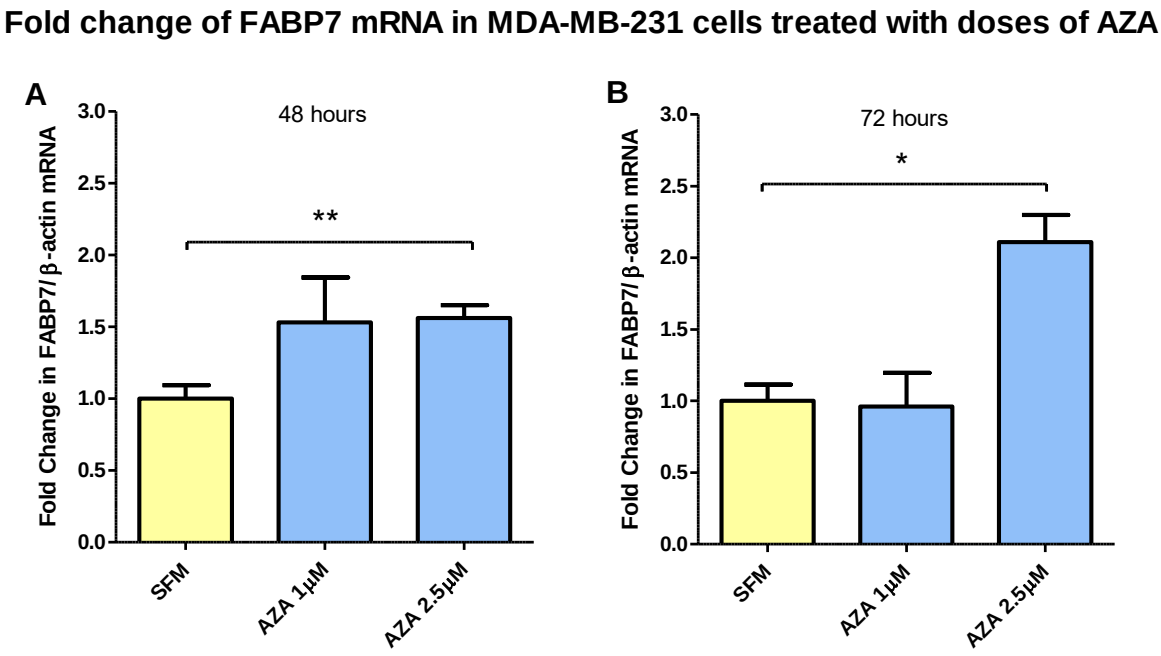


Figure 4.3 Fold Change of FABP7 mRNA in MDA-MB-231 cells treated with 2 different doses of AZA

Results shown are expressed as fold change relative to FABP7 mRNA in cells in SFM alone. A) Fold change in FABP7 mRNA after 48 hours 1μM (1.53 fold change) or 2.5μM **(1.56 fold change p=0.0073) AZA treatment. B) Fold change in FABP7 mRNA after 72 hours 1μM (0.96 fold change) or 2.5μM *(fold change of 2.11 p=0.0167) AZA treatment. Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

4.4.3 FABP7 mRNA expression changes in BT-20 cells after treatment with TSA or AZA

BT-20 cells were treated with 0.25μM and 0.5μM TSA for 24 hours and 1μM or 2.5μM AZA for 24, 48 and 72 hours (figure 4.4). A dose of 0.5μM TSA (A) gave a 1.6 fold increase in FABP7 mRNA although this was not statistically significant. A significant increase in FABP7 mRNA with AZA treatment was only present after 72 hours exposure (D), 1μM gave a 1.83 fold increase (p= 0.0212); 2.5μM gave the largest fold increase of 2.19 (p=0.0001). There was not any observable change in cell death as a result of AZA or TSA treatment.

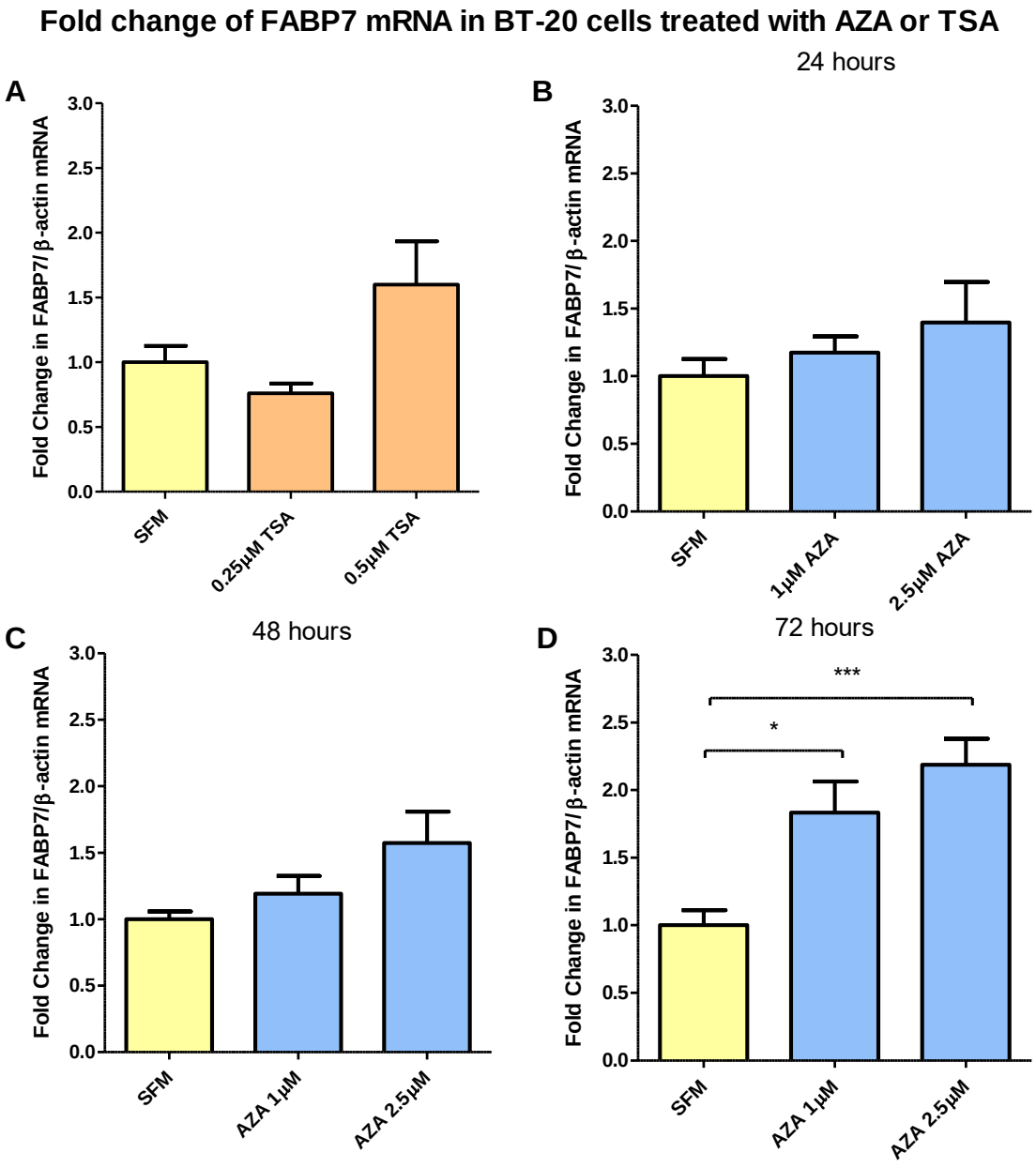


Figure 4.4 Fold change of FABP7 mRNA in BT-20 cells treated with TSA or AZA

Results are shown as fold change in FABP7 mRNA relative to FABP7 mRNA in BT-20 cells in SFM alone. A) Fold change in FABP7 after TSA treatment for 24 hours, 0.25µM gave a 0.76 fold change and 0.5µM gave a 1.6 fold increase. B) Fold change in FABP7 mRNA after 24 hours 1µM AZA (1.17 fold change) or 2.5µM (1.40 fold change) AZA treatment. C) Fold change in FABP7 mRNA after 48 hours 1µM (1.19 fold change) or 2.5µM (1.57 fold increase) AZA treatment. D) Fold change in FABP7 mRNA after 72 hours AZA 1µM *(1.83 fold increase p=0.212) or 2.5µM ***(2.19 fold increase p=0.0001) AZA treatment. Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

4.4.4 AZA and TSA combined increase FABP7 mRNA expression

Since the biggest increase in FABP7 mRNA was noted in the BT-20 cell line after 72 hours and as it appeared that dose of 0.5µM TSA could increase FABP7 mRNA; BT-20 cells were treated with both 2.5µM AZA for 72 hours and 0.5µM TSA for the final 24 hours. Figure 4.5 shows that a 2.73 fold increase in FABP7 mRNA resulted (p=0.0001) compared to TSA (1.4 fold) and AZA alone (1.9 fold p=0.0125).

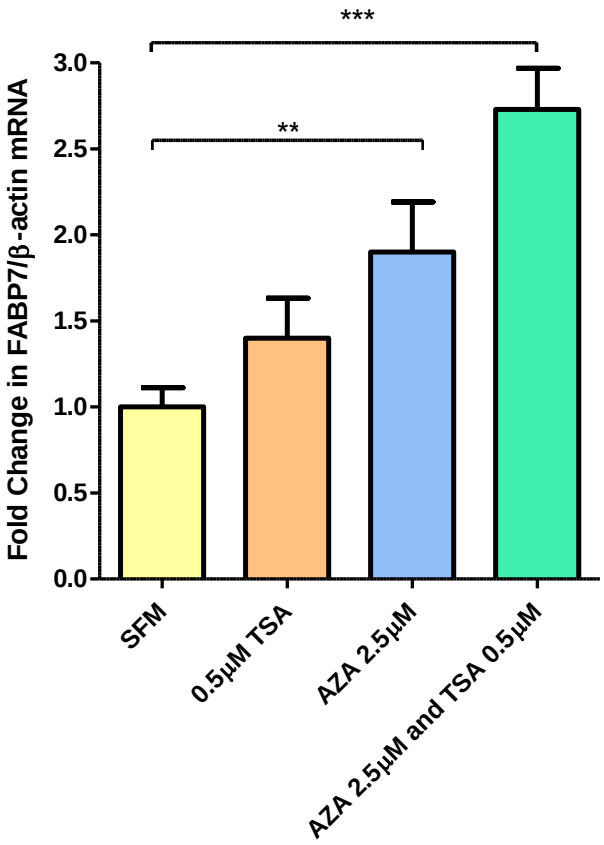


Figure 4.5 Fold change in FABP7 mRNA in BT-20 cells after treatment with TSA and AZA together
Results are shown as fold change in FABP7 mRNA relative to FABP7 mRNA in BT-20 cells in SFM alone. BT-20 cells were treated with 2.5µM AZA for 72 hours, 0.5µM TSA was added for the final 24 hours. There was 2.73*** fold increase in FABP7 mRNA (p=0.0001) relative to cells in SFM alone and compared to TSA (1.4 fold) and AZA alone **(1.9 fold p=0.0125). Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

4.4.5 Fatty acid treatment and FABP7 mRNA expression in MDA-MB-231 cells

MDA-MB-231 cells were treated with doses of oleate palmitate, AA and DHA. Figure 4.6 shows FABP7 mRNA expression after 24 hours (A) and 48 hours (B). The only statistically significant change was with 10µM AA for 24 hours and this was a fold decrease of 0.47 (p=0.0152). The same trend was present at 48 hours.

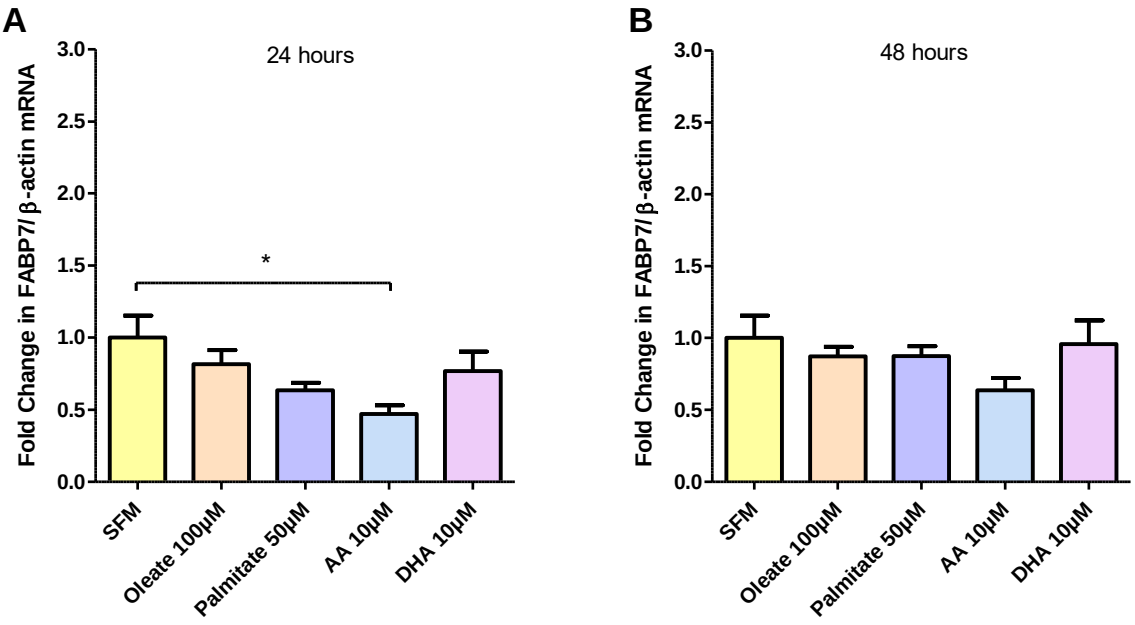


Figure 4.6 Fold change in FABP7 in MDA-MB-231 cells after treatment with fatty acids

Results are presented as fold change in FABP7 mRNA relative to FABP7 mRNA in cells in SFM Resulting fold changes at 24 hours are illustrated in A and are as follows: oleate 0.81, palmitate 0.63, AA 0.47 *(p=0.0152) and DHA 0.77. Fold changes at 48 hours are shown in B and are as follows: oleate 0.87, palmitate 0.87, AA 0.63 and DHA 0.96. Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

4.4.6 Fatty acids alter expression of FABP7 mRNA in BT-20 cells

BT-20 cells were treated with oleate, palmitate, AA or DHA; results are in figure 4.7 for 24 hours (A) and 48 hours (B). After 24 hours exposure of oleate there was a small but significant increase in FABP7 mRNA by 1.2 fold (p=0.0291), however after 48 hours oleate treatment led to a reduction in FABP7 mRNA by 0.69 fold (p=0.0151). DHA increased FABP7 mRNA by 1.31 fold (p=0.0413) after 24 hours but no significant changes were present at 48 hours. Palmitate also increased FABP7 mRNA but significant increases were not present until after 48 hours (1.38 fold p=0.0350).

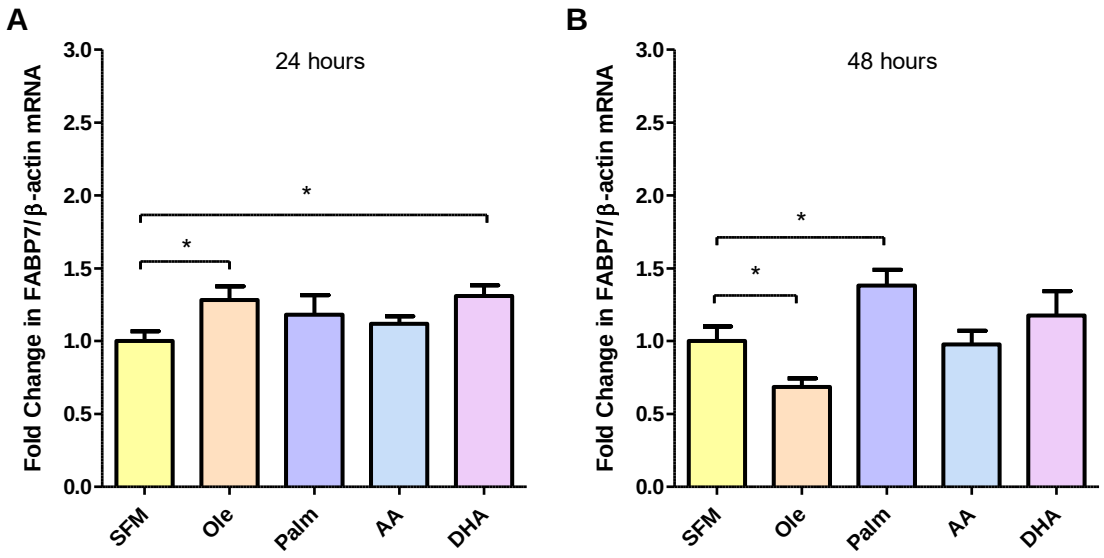


Figure 4.7 Fold change in FABP7 in BT-20 cells after treatment with fatty acids

Results are shown as fold change in FABP7 mRNA relative to FABP7 mRNA in BT-20 cells in SFM alone BT-20 cells were treated with either oleate, palmitate, AA or DHA. Results are presented as fold change in FABP7 mRNA relative to FABP7 mRNA in cells in FA free BSA. Resulting fold changes at 24 hours are illustrated in A and are as follows: oleate 1.28*(p=0.0291), palmitate 1.18, AA 1.12 and DHA 1.31 *(p=0.0413). Fold changes at 48 hours are shown in B and are as follows: oleate 0.69 *(p=0.0151), palmitate 1.38 *(p=0.0350), AA 0.98 and DHA 1.18. Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.

4.4.7 AZA and combined FA treatment alter the expression of FABP7 mRNA in BT-20 cells

BT-20 cells were treated with 2.5µM AZA for 48 hours and then treated with each of the FAs for a further 24 hours with a second dose of AZA. The second dose of 2.5µM AZA gave the highest fold increase than previous AZA alone treatments (140 fold ($p=0.0005$). AZA and palmitate together gave the biggest fold increase of 249 ($p=0.0005$). Oleate, AA and DHA with AZA gave bigger fold changes than alone but were less than the fold increase of AZA alone; 65.5, 95 and 35 respectively. The results of this experiment are show in figure 4.8.

4.4.8 AZA combined with palmitate treatment increases FABP7 protein in BT-20 cells

Next the expression of FABP7 protein was assessed in response to AZA and palmitate treatment as this combination had increased FABP7 mRNA expression the most compared to other AZA and FA combinations (figure 4.9). The relative band intensities indicate that combined treatment of BT-20 cells with AZA and palmitate gave the largest increase in FABP7 protein expression.

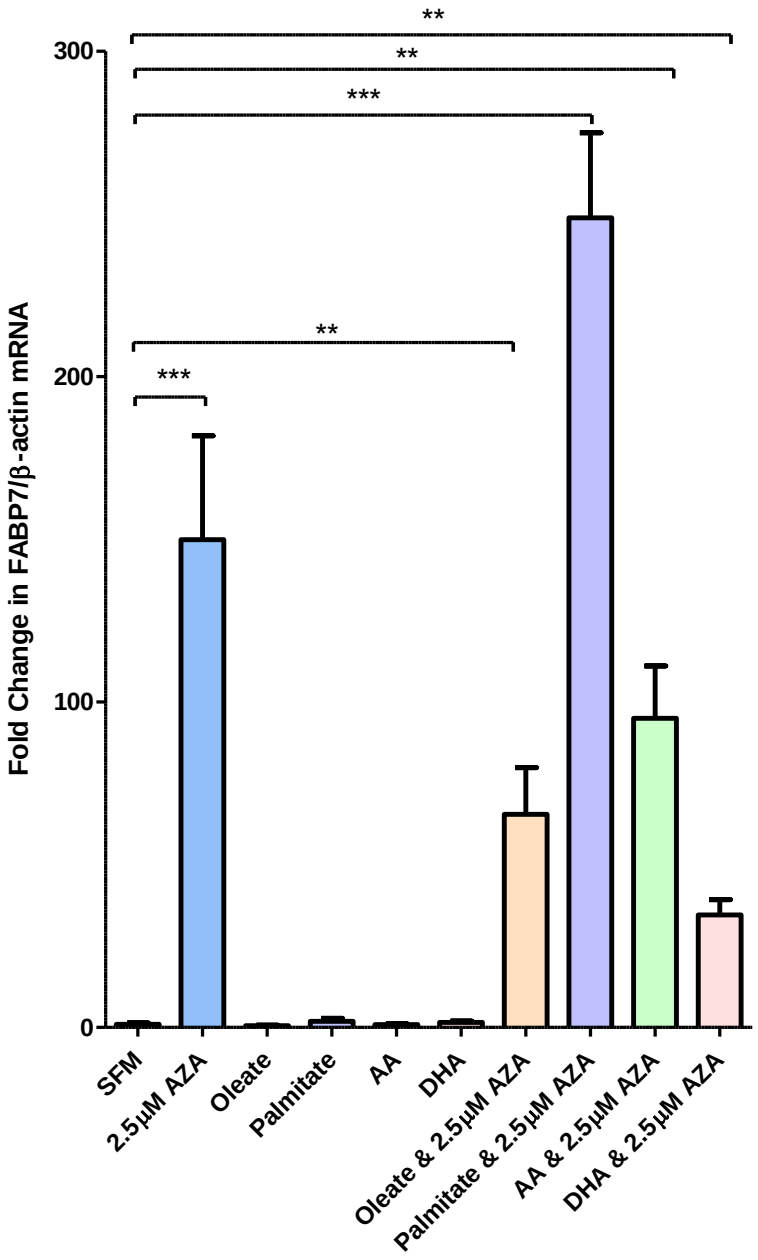
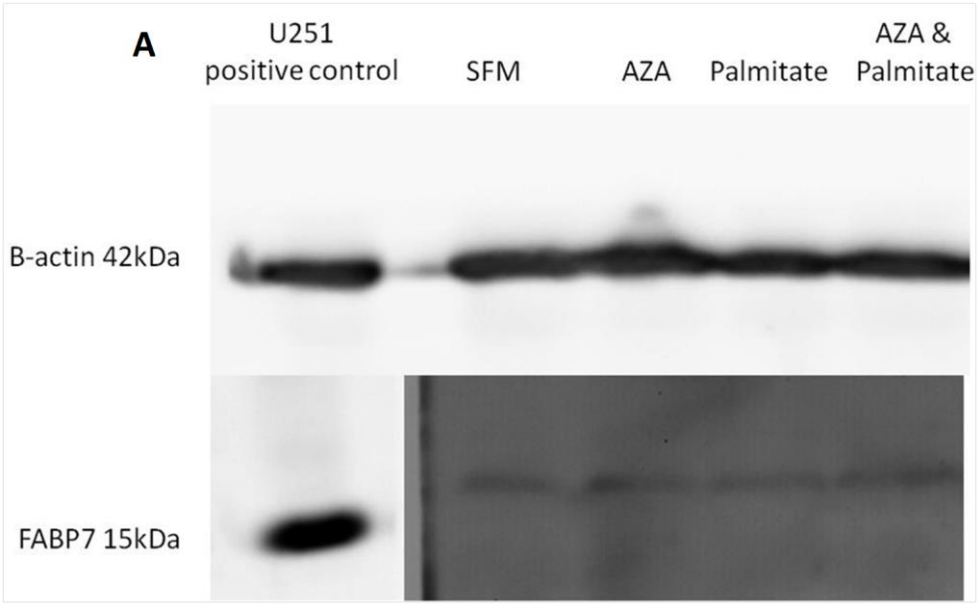


Figure 4.8 The expression of FABP7 mRNA in BT-20 cells treated with FAs and AZA combined

Results shown are fold change in FABP7 mRNA expression relative to FABP7 mRNA in BT-20 cell in SFM alone. The fold changes for the FA alone treatments were in line with those seen in figure 4.6. Two doses of AZA gave a fold increase of 140 ***(p=0.0005). The fold changes for the AZA and each of the following FAs were: oleate 65.5 **(p=0.0040), palmitate 249 ***(p=0.0005), AA 95 **(p=0.0038) and DHA 35 **(p=0.0013). Data represent the mean of 3 independent experiments carried out in triplicate ±SEM.



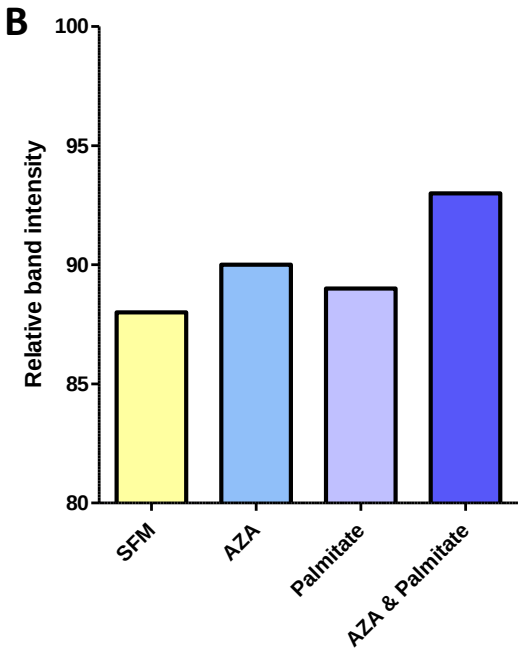


Figure 4.9 Western blot showing FABP7 protein in untreated cells, palmitate and AZA alone and combined treated cells

BT-20 cells were treated with AZA, palmitate and both combined, and then lysed in sample buffer and a western blot performed. A) The cell line U251 was used as a positive control for FABP7 and imaged separately; B-actin was used as a loading control. B) The relative intensities of each band were analysed by ROI manager in Image J. AZA and palmitate combined gave the highest band intensity followed by AZA alone; this indicates that there is more FABP7 protein present. This figure represents one repeat of three.

4.5 Discussion

As epigenetic mechanisms and FAs have been shown to regulate FABPs, MDA-MB-231 and BT-20 cells were treated with AZA, TSA and FAs to determine whether FABP7 is regulated by such mechanisms in breast cancer cell lines

4.5.1 ER mRNA expression is increased in BT-20 and MDA-MB-231 cells with AZA and TSA

To demonstrate that TSA and AZA could alter gene expression, PCR was carried out to look for ER re-expression as ER has been shown to be methylated and affected by acetylation in these cell lines (Ferguson *et al.*, 1997; Yang *et al.*, 2000; Yang *et al.*, 2001). ER mRNA increased significantly with AZA treatment up to 32 and 33 fold in MDA-MB-231 and BT-20 cells respectively. ER was increased by TSA to 6.1 fold in MDA-MB-231 cells and to 8.1 fold in BT-20 cells. The increases in ER expression in both cell lines with both treatments were similar to the fold changes reported by Yang *et al.* (2001) They treated MDA-MB-231 and MDA-MB-453 cells with 2.5µM AZA for up to 96 hours; this resulted in a 31 fold increase in ER mRNA in MDA-MB-231 cells and a 36 fold increase in the MDA-MB-453 cells.

4.5.2 AZA significantly increases FABP7 mRNA expression

The increases with the highest doses of AZA at 72hours in both cell lines were significant though not as high as expected. If FABP7 is methylated in the cell lines used in the current study then the fold increases would have been more marked perhaps similar to the level of ER mRNA increases. Longer time points and higher doses could have been investigated further though the half-life of AZA is relatively short and higher doses are likely to have resulted in toxicity based on pre-published experiments by collaborators at the University of Bristol using AZA and TSA on breast cell lines. The time points used would have been sufficient to take into account cell turnover and

the half life of the drug so duration was not increased further. The FABP7 promoter was analysed for CpG islands (using bioinformatics.org) and none were found; this is in agreement with Goto *et al.*, (2010); however the experiments were still required as methylation could occur at regions other than the promoter, at histones or at regions other than CpG islands such as so called "weak CpG regions" (Meissner *et al*., 2008). One study found that AZA causes re-expression of genes though demethylation of histones in particular H3 residues (Wozniak *et al.*, 2007). An alternative is that the increases in FABP7 mRNA were a secondary effect of AZA treatment; for example global expression of genes was studied after treatment of cells with AZA. It was found that 116 genes were up-regulated by around four fold by such treatment but methylation of DNA was not connected; suggestions for this included demethylation of histones, increased RNA stability and changes in regulatory genes to give a knock on increase of the particular genes of interest (Shi *et al.*, 2003). The signal transducer and activator of transcription (STAT) transcription factors have been shown to be up-regulated in response to AZA treatment; there are CpG islands that could have been methylated or alternatively the increase could have been a result of demethylation of an upstream regulator of STATs *(*Karpf *et al.*, 1999). Interestingly STAT3 is a potential transcription factor of FABP7, the FABP7 promoter has three STAT3 binding sites; (identified in genecards) moreover STAT3 is important in mammary gland development and breast cancer cell proliferation; breast tumours that expressed both activated STAT5 and STAT3 were more differentiated than those with activated STAT3 alone (Walker *et al.*, 2009). STAT3 has also been found to be methylated at histones (Yang *et al.*, 2010). For these reasons it is probable that FABP7 is indirectly increased by AZA treatment, perhaps by a transcription factor such as STAT3 that is methylated at either CpG islands or at histones; other transcription factors identified in genecards include nuclear factor-I (NF-I) and ETS related gene-4 (ERG-4) and could equally altered by epigenetic mechanisms to increase FABP7 mRNA.

4.5.3 TSA treatment did not increase FABP7 mRNA expression

Treatment with TSA did not significantly increase FABP7 mRNA with either dose in both cell lines. As with treatment with AZA, the effects may be indirect, treatment only occurred for 24 hours so indirect increases in FABP7 mRNA may not have had chance to occur. STAT3 is hyperacetylated in many breast tumours and as a result is over-expressed; inhibiting HDACs with TSA increased methylation of tumour suppressor genes, such as p53, through STAT3 interacting with DNMT1 (Lee *et al.*, 2012). This shows that transcription factors and substances such as TSA can have opposing effects on epigenetic alterations of gene expression at the same time. It is possible that such mechanisms maybe involved in the expression of FABP7. A study by Van Lint *et al.* (1996) demonstrated that of 340 genes tested only the expression of 8 genes were changed in response to treatment with TSA or another HDAC inhibitor trapoxin. Considering that hyperacetylation of histones is global it was surprising that the expression of so few genes was altered; they considered that the action of HDAC inhibitors may be more specific and that some histones are more sensitive to hyperacetylation than others, possibly due to differences in the microenvironment (Van Lint *et al.*, 1996). This could be a reason as to why FABP7 mRNA expression was not altered significantly by TSA treatment. Another important point was that changes in gene expression may be secondary to HDAC inhibitor treatment i.e. that change in expression is a result of the change in expression of another gene that is directly affected by TSA (Van Lint *et al.*, 1996). In light of this it is unlikely that the FABP7 gene is altered directly by acetylation. Increasing the dose or duration of TSA would have had significant cytotoxic effects on the breast cell lines so it is not feasible with the current method to study longer term effects of TSA on FABP7 expression.

4.5.4 Combined AZA and TSA treatment resulted in an increase in FABP7 mRNA expression that was higher than that induced by either drug alone.

Synergism is defined as the working together of two 'things' to produce an effect that is greater than the sum of their individual effects. Using AZA and TSA together further increased FABP7 mRNA in BT-20 cells, than either used alone; it is likely that this is the result of the two substances combined rather than them having a synergistic action. For example the ER was expressed in breast cell lines to a level of around 400 fold after treatment with AZA and TSA together, each on their own gave a 30-40 and five fold increase respectively (Yang *et al.*, 2001).

4.5.5 FA treatment altered expression of FABP7 mRNA

It was decided to investigate whether FAs could regulate FABP7 expression; each FA was chosen due to their association with FABP7 and the evidence that they regulate other FABPs; the FAs also covered a range of chain lengths and saturation differences. Differences in chain length and three dimensional structure of the FAs as a result of saturation differences could inhibit or promote binding of transcription factors or co-factors. The FA treatments resulted in different changes in FABP7 mRNA expression between the cell lines. Reasons for this are that BT-20 cells have more FABP7 mRNA than MDA-MB-231 cells to begin with and that signalling pathways may be activated at a different level in each cell line by the FA. The only statistically significant change in FABP7 mRNA in the MDA-MB-231 cells was after 24 hours of treatment with 10µM AA resulting in a decrease of 0.47 fold. Palmitate also decreased FABP7 mRNA at the same time point but this was not significant. Interestingly AA has been well documented to decrease gene expression. For example 3T3-L1 pre-adipocyte cells were treated with AA and then GLUT4 mRNA analysed; not only did the amount GLUT4 mRNA transcription decrease over time but so did the stability of the mRNA leading to near total protein reduction (Tebbey *et al.*, 1994); little work has been done since looking at FAs and mRNA expression. In addition to this AA decreased the enzymatic activity

of stearoyl-CoA desaturase and its mRNA expression (Sessler & Ntambi, 1998). These two studies looked at mRNA expression and stability of GLUT4 or stearoyl-CoA desaturase at time points from 30 minutes to 48 hours post FA treatment. The genes were decreased at different time points (Tebbey *et al.*, 1994; Sessler & Ntambi, 1998). In the current study if FABP7 mRNA expression was measured at time points before 24 hours then perhaps a gradual reduction in expression could be seen followed by an increase in FABP7 mRNA between 24 and 48 hours where FABP7 mRNA expression was comparable to the control. After 48 hours palmitate increased FABP7 mRNA by 1.38 fold in BT-20 cells, the opposite of what was noted in MDA-MB-231 cells. This increase of FABP7 mRNA in BT-20 cells with palmitate is in conjunction with another study; it was noted that of the FAs tested palmitate produced the greatest increase of mRNA of FABP3 and it was postulated that this was due to accumulation of palmitate in the cytoplasm inhibiting fatty acid oxidation (Chang *et al.*, 2001). This does not explain why palmitate resulted in opposing effects on FABP7 mRNA expression in each cell line; however, palmitate has been shown to both increase and decrease gene expression. For instance, in rat islet cells it can inhibit preproinsulin levels through its conversion to ceremide (Kelpe *et al*., 2003). In contrast to this palmitate induces interleukin-6 mRNA expression and NF-κB activation in skeletal muscle cells (Weigert *et al*., 2004). The opposing effects of palmitate could be explained by different pathways being active in each of the tissue types and in the present study, the breast cell lines. The difference in effect could be due to how much FABP7 mRNA is present basally or how much palmitate accumulates in the cells. What is intriguing is that oleate treatment in BT-20 cells initially increased FABP7 mRNA relative to untreated cells at 24 hours and then levels dropped significantly after 48 hours. One potential reason for this is the mRNA being translated into protein and subsequently degrading beyond 24 hours. Considering the 48 hour time point on its own it is clear that oleate and palmitate have opposing effects on FABP7 mRNA expression; such opposing effects of these 2 FAs have been documented in other studies. Oleate has been demonstrated to activate *PI3K* and in turn proliferation whereas palmitate has the opposite effect in MDA-MB-231 cells (Hardy *et al.*, 2000);

this investigation also found that oleate blocks the effects of palmitate. Another study looked at gene transcription after oleate or palmitate treatment in HepG2 cells; palmitate up-regulated 724 genes and suppressed 1035 and oleate induced 1669 and down-regulated 1535 genes; of these genes there were only 417 genes that were up-regulated and 452 down-regulated by both oleate and palmitate (Das *et al.*, 2001). However this does not explain why oleate initially increased FABP7 mRNA, one possible reason is that the BT-20 cells are adapting to the oleate treatment; they respond by initially increasing production of FABP7 but then a feedback mechanism may be activated leading to a reduction in FABP7 production. This increase by oleate treatment is in conjunction with FABP1 being up-regulated at both the mRNA and protein level by oleate in rat hepatoma cells (Meunier-Durmort *et al.*, 1996); very little work has been done since looking at changes in FABP expression as a result of exposure to FAs. The other significant change in FABP7 mRNA in BT-20 cells was with DHA after 24 hours resulting in an increase in FABP7 mRNA. Of note is that DHA activates the RXR by acting as a transcription factor when bound to FABP7; however this causes the activation of the receptor rather than FABP7 itself but it is plausible that there may be some feedback mechanism involved that in turn alters the expression of FABP7 (Liu *et al.*, 2012). This is also indicative that FAs can have secondary effects resulting in changes in FABP7 mRNA. For instance, FAs could activate or increase expression of transcriptions factors or deactivate and prevent expression of inhibitors of FABP7 expression. Another possibility is that transcription factors could be recruited to the promoter site FABP7 as a result of FA treatment; as palmitate treatment has been found to increase binding of various transcription factors to the Glucose 6-phophatase gene promoter (Xu *et al.*, 2007).

4.5.6 Combinations of AZA and FA increase FABP7 mRNA in BT-20 cells

A combination of AZA and the FAs were used to treat BT-20 cells to investigate whether there was any synergistic action as FAs and AZA altered FABP7 mRNA expression individually. The second

dose of AZA considerably increased FABP7 mRNA compared to the first experiments with AZA alone, this gives further indication that the action is indirect; a second dose would ensure many pathways upstream of FABP7 would be highly activated within a shorter time compared to one dose of AZA, resulting in further up-regulation of FABP7 within the 72 hour time point. Moreover, treatment with AZA and palmitate resulted in the highest observed fold increase of FABP7 mRNA in the present study, significantly more than AZA alone and palmitate alone fold changes combined together. As discussed there is evidence that palmitate increases binding of transcription factors to promoter regions; it is possible that AZA results in demethylation of genes that regulate FABP7 and transcription of those genes is increased with palmitate treatment. An alternative is that palmitate may alter mRNA stability or activity of transcription factors. Intriguingly, oleate, AA and DHA treatment with AZA significantly reduced the expression of FABP7 mRNA than the 2 doses of AZA alone. Oleate decreasing FABP7 mRNA after AZA treatment is in agreement with previous results showing oleate decreases FABP7 mRNA when used individually to treat BT-20 cells; this could be reflective of oleate and palmitate having opposite effects. There was no significant change with AA treatment alone and only a small significant increase with DHA alone as seen in the previous experiment at 24 hours. One reason DHA may decrease FABP7 mRNA after AZA treatment, relative to two doses of AZA alone, is because there was very little FABP7 mRNA present basally for AA and DHA to alter expression. The increase in FABP7 mRNA expression as a result of AZA could have allowed the actions of AA and DHA to be more marked.

4.5.7 AZA and palmitate increase FABP7 protein expression in BT-20 cells

To ascertain whether there were changes in protein expression following on from the AZA and FA treatment, a western blot was carried out on lysates from AZA alone and palmitate combined with AZA treated BT-20 cells, as these treatments resulted in the greatest fold increases in FABP7

mRNA. Band densitometry normalised to β-actin was carried out as it was hard to see subtle changes in relative amount of FABP7 protein by eye. There was an increase in protein by AZA alone and AZA and palmitate treatment combined in conjunction with the increase in FABP7 mRNA. It is surprising that there was not a greater increase in protein considering the fold changes in FABP7 mRNA. There were a number of potential reasons for this; mRNA expression does not always link to protein expression and this could be due to protein or mRNA degradation or a delay in protein being produced. It would be hard to postulate whether the increase in FABP7 mRNA is enough to physiologically affect the cells and in addition the effects of AZA, TSA and FAs are not specific to FABP7.

4.6 Conclusions

It is unlikely that FABP7 is directly regulated by methylation and acetylation; changes in FABP7 mRNA are likely to reflect alterations in genes upstream of FABP7. Treatment of TN breast cell lines BT-20 and MDA-MB-231 with FAs resulted in small increases and decreases in FABP7 mRNA; this could be a result of direct or indirect mechanisms. Palmitate and AZA synergistically increased FABP7 mRNA and protein levels.

5 FABP7 over-expression in the triple negative breast cancer cell line BT-20

5.1 Introduction

To assess the functional relevance of FABP7 in breast cancer pathology, FABP7 was transiently over-expressed in BT-20 cells. The effects of FABP7 over-expression on cell growth and viability was investigated. In addition to this expression of pro/anti apoptotic genes (bcl-X, caspase 9 and survivin) were assessed in order to investigate the influence of FABP7 expression on cell survival. Bcl-x is related to the bcl-2 gene and functions as an independent, dominant regulator of apoptosis and two distinct splice variants have been identified (Boise *et al.*, 1993); bcl-x_L, the larger variant and bcl-x_S, the smaller variant. Blc-x_L is an anti-apoptotic splice variant whilst bcl-x_S is pro-apoptotic (Mercatante *et al.*, 2001). In the prostate cancer cell line PC3 apoptosis can be induced by shifting splicing from bcl-x_L to bcl-x_S, however reducing bcl-x_L levels but keeping bcl-x_S levels constant was less efficient at inducing apoptosis. In addition increasing bcl-x_S in the breast cancer cell line MCF-7 reduced survival but not to the same extent as in PC3 cells (Mercatante *et al.*, 2001). Changes in bcl-x splicing have also been found to alter tumour growth in xenografts (Bauman *et al.*, 2010). As bcl-x protein has been reported to be over-expressed in around 40% of invasive breast cancers (Olopade *et al.*, 1997), changes in splicing were investigated in relation to FABP7 over-expression.

Caspase 9 is an initiator of apoptosis; there are also 2 splice isoforms, caspase 9A, longer and pro-apoptotic and caspase 9B, shorter and anti-apoptotic (Srinivasula *et al.*, 1999; Massiello & Chalfant, 2006). One study found that down-regulating the splice factor SRp30a decreased caspase 9A and increased caspase 9B (Massiello & Chalfant, 2006). Another regulator of caspase 9 splicing is hnRNPL, down-regulating it was found to decrease the caspase 9A:9B ratio and in turn decrease tumourigenicity (Goehe *et al.*, 2010). Studying changes in the ratio of caspase 9 splice

isoforms in relation to FABP7 transfection gives information on the apoptotic state that cells are in. Survivin is an inhibitor of apoptosis protein (IAP) with 2 splice isoforms, survivin ΔEx3 and survivin 2B. Survivin ΔEx3 exhibits anti-apoptotic properties similar to that of full length survivin whereas survivin 2B has a portion of intron 2 retained and has reduced anti-apoptotic abilities (Mahotka *et al.*, 1999; Badran *et al.*, 2004). It has been suggested that survivin 2B is a natural antagonist to survivin ΔEx3 and full length survivin and its expression may be favourable with its anti-tumourigenic properties (Li, 2005). Moreover full length survivin protein expression was found to be expressed in around 70% of breast cancers; when combined with the apoptotic index of the tumour the results suggested that inhibition of apoptosis by survivin is a poor prognostic factor (Tanaka *et al.*, 2000).

Bcl-x, caspase 9 and survivin and their splice isoforms were relevant because they give information on both the pro- and anti-apoptotic features of the cells and therefore provide further *in vitro* evidence as to the likely effect of FABP7 expression in breast cancer cells. In addition this study examined whether FABP7 over-expression in BT-20 cell line conferred resistance or sensitivity to cell death by various insults. For example staurosporine (STRP) is a bacterial alkaloid that inhibits a range of protein kinases; STRP induces apoptosis but the pathways through which it does this are not fully understood, it is thought that there are several mechanisms by which STRP may exert its action (Weil *et al.*, 1996; Yoo et al., 2012). STRP has been successfully used in other studies to treat BT-20 cells and evaluate sensitivity to cell death after other treatments. For example STRP induced cell death was reduced by treatment of cells with IGF-I (Davison *et al.*, 2011); STRP was also used to demonstrate that a tyrosine kinase, FAK, prevented cell death by binding to a death domain of receptor interacting protein (RIP). STRP induced apoptosis caused degradation of FAK that was RIP dependent as STRP caused a higher amount of apoptosis in RIP positive cells than negative (Kurenova *et al.*, 2004). Due to the apoptotic properties of STRP, it was chosen to treat BT-20 cells post over-expression of FABP7. As well as this it was informative to know whether over-expression of FABP7 altered percentage cell

death with treatment of FAs; as FABP7 binds oleate, palmitate, AA and DHA; it was hypothesised that FABP7 transfected BT-20 cells would respond differently to treatment with these FAs, either shuttling fats to increase death or act as a sink and stopping signalling induced by fats to prevent death. Oil red O staining was used in addition to counting cells to look for changes in FA accumulation. These investigations may give an indication of the role of FABP7 in breast cancer cell lines and their survival and growth

5.2 Aims and hypotheses

5.2.1 Aims

i) To investigate whether FABP7 expression influences cell death

ii) To investigate whether FABP7 affects FA accumulation in BT-20 cells.

5.2.2 Hypotheses

i) FABP7 will alter sensitivity to cell death by treatment with FAs and STRP

ii) FABP7 will alter the expression of bcl-x, caspase 9 and survivin and their splice isoforms.

5.3 Materials and Methods

5.3.1 Tissue culture and dosing experiments

BT-20 cells were seeded at a density of $0.5x10^6$ per well of a 6 well plate. Cells were transfected as described in section 2.8. Non-transfected cells, FugeneHD alone treated and control vector transfection cells were used as controls in these investigations. To study cell death and growth, cells were transfected as detailed previously and then trypsinized and counted following trypan blue staining (section 2.3.9) after 24, 48 and 72 hours. In addition to this, 24 hours post transfection cells, were trypsinized and seeded at 6000 cells per well of a 96 well plate. The following morning cells were subjected to an MTT assay within the 48 hour time period, to assess cell viability as described in 2.9. For the FA and cell death experiments transfected BT-20 cells were treated as described in section 2.3 with 2 doses of each FA chosen on the results of the dose response in section 1.10 and also on the results of work carried out by Liu *et al.* (2012). The doses for the FAs were as follows; palmitate 50µM and 200µM, oleate 50µM and 100µM, AA 10µM and 50µM and DHA 10µM and 50µM. FAs were given 24 hours post transfection and incubated with the cells for a further 24 hours. For the STRP experiments, cells were transfected and then medium changed to SFM 45 hours post transfection; this time point was chosen because the STRP treatment totalled 3 hours meaning that cells were treated and collected within 48 hours post transfection. After one hour 1µM of STRP was spiked into the medium; two hours later the dead cells were collected and the live trypsinized and re-suspended together for counting (section 2.3.9). The dose and time of STRP incubation were chosen based on previous optimisation work; cells were treated with doses of STRP ranging between 0.25µM and 2µM for between one and three hours.

5.3.2 Sequencing of FABP7 pCMV.SPORT6 vector

The plasmid was sent according to instructions using pre-paid barcodes to Eurofins MWG Operon DNA sequencing service. It was sequenced in both directions using T7 and SP6 primers. This verified that the plasmid contained the correct and complete FABP7 gene.

The results of the sequencing were then aligned with the FABP7 sequence (accession NM_001446.3). The alignment was done using EMBL-EBI ClustalW2 online program

5.3.3 Generation of an empty pCMV.SPORT6 vector was generated for use as a control

The following procedure is outlined in detail in section 2.8. To generate the empty vector the FABP7 pCMV.SPORT6 vector was subjected to a double restriction enzyme digest. The larger band of the double digest was extracted and purified and then religated. The religation product was transformed into competent JM109 cells which were then cultured and subjected to plasmid prep as described. To verify that it was an empty pCMV.SPORT6 vector that had been cultured it was subjected to a single and double digest alongside the FABP7 containing vector with SMAI and HINDIII.

5.3.4 RNA extraction, PCR and gel densitometry

The TRI reagent method as detailed in section 2.6.2 was utilised for RNA extraction, as this method gave higher yield. For initial experiments RNA was collected 24, 48 and 72 hours post transfection. Reverse transcription, standard and qPCR took place as in section 2.7. The standard PCR products were imaged and semi-quantified using densitometry by measuring band intensity in ImageJ. Briefly, the region of interest tool was selected and exactly the same size box was drawn around each band; the program then generated arbitrary numbers based on the intensity of each band. B-actin was checked to ensure even sample loading. The ratios of the bands were

calculated for each sample again to ensure loading was equal and to visualize changes in apoptotic potential. All results for the mRNA expression of bcl-x, survivin and caspase, are presented so the larger the expression or ratio on the graph the lower the apoptotic potential/higher anti-apoptotic potential gene expression. qPCR was used to identify the time point with the biggest increase in FABP7 mRNA and to confirm results from the standard PCR densitometry. Again the ratio of the splice variant expression was calculated to see differences in pro- or anti-apoptotic properties.

5.3.5 Western Blotting

Protein extraction and western blotting was carried out as detailed in section 2.5. Protein lysates were made 24, 48 and 72 hours post transfection to identify suitable time points for further work.

5.3.6 Statistical Analysis

All statistics were carried out on the results of 3 independent experiments that had been carried out in triplicate unless otherwise stated. The statistical package Graphpad Prism 5 was used to produce graphs and carry out statistical analyses for these experiments. Whenever there was more than one comparison against the control a one-way ANOVA with a Bonferroni post-hoc test was used to highlight differences; a students paired t-test was then carried out to confirm significant differences. Data are presented as mean ± standard error of the mean (SEM).

5.4 Results

5.4.1 T7 primer vector sequencing

GTACGCGTAGCTTGGGCCCCTCGAGGGATACTCTAGAGCGGCCGCCCTTTTTTTTTTTTTTCACGTGTAAG
ATTTTTATTCAAATTTGATTTACATTCCAAAAGAAATTATAAAATGTATTCACTTGTTTATAAAAAAAAGGGG
GGGGGGACAAAACTTTAATTCAAATTATAAAACATGATAAATTTTCAGATTAAAATTGGGCAAGTTGCTTG
GAGTAACAAGTTTTTAAATCACCATTTTCCACCTCCACACCAAGGATAACCTTCTAATTAATGATCAGCCATG
TTGTAATAGGATAGCACTGAGACTTGAGGAAACAGAAAAACTGAAGAGCTCTTCCAAGCCCCGACCAGGA
ACATTTTTATGCCTTCTCATAGTGGCGAACAGCAACCACATCACCAAAAGTAAGGGTCATAACCATTTTGCC
ATCCTTAATTTCTCTTACAAAATTTGTTTCTTTGCCATCCCATTTCTGTATGTGAACAAGTTTGTCTCCATCCA
GGCTAACAACAGACTTACAGTTTCTATCATCTGCAGTGGTTTCATCAAACTCTTCTCCCAGCTGGAAACTAA
TCTCCGTGTTCTTGAATGTGCTGAGAGTCCTGATGACCACTTTGTCTCCTTCTTGACTGATAATTACCGTTGG
TTTGGTCACATTTCCCACCTGCCTAGTGGCAAAGCCCACGCCTAGAGCCTTCATGTACTCATCAAAGTTCTG
ACTGTTGGTCAGCTTCCAGGTAGCACAGAAAGCCTCCACCATCCTTGCCCTTTCCCCTCTTTAGAGACAGGA
GCGGGGATCTTCTGGTCTAATTGCCACTGCAGCTCAGAAGACCCTTTACACCTCAGCAAGCAGCTCCTGCCT
TCTTATTTGGAAAGAGGGTGGGCAAAAATCCAGTGATTTAAATTGCAAACACACCCCTCCAGTGTCCCTCTT
TCCAAGAGCTGCGGACGCGTGG

5.4.2 SP6 vector sequencing

GCAGGCTGGTACCGGTCCGGATTCCCGGGATATCGTCGACCCACGCGTCCGCAGCCTCTTGGAAAGAGGG
ACACTGGAGGGGTGTGTTTGCAATTTAAATCACTGGATTTTTGCCCACCCTCTTTCCAAATAAGAAGGCAG
GAGCTGCTTGCTGAGGTGTAAAGGGTCTTCTGAGCTGCAGTGGCAATTAGACCAGAAGATCCCCGCTCCTG
TCTCTAAAGAGGGGAAAGGGCAAGGATGGTGGAGGCTTTCTGTGCTACCTGGAAGCTGACCAACAGTCAG
AACTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCAGGTGGGAAATGTGACCAAAC
CAACGGTAATTATCAGTCAAGAAGGAGACAAAGTGGTCATCAGGACTCTCAGCACATTCAAGAACACGGA
GATTAGTTTCCAGCTGGGAGAAGAGTTTGATGAAACCACTGCAGATGATAGAAACTGTAAGTCTGTTGTTA
GCCTGGATGGAGACAAACTTGTTCACATACAGAAATGGGATGGCAAAGAAACAAATTTTGTAAGAGAAAT
TAAGGATGGCAAAATGGTTATGACCCTTACTTTTGGTGATGTGGTTGCTGTTCGCCACTATGAGAAGGCAT
AAAAATGTTCCTGGTCGGGGCTTGGAAGAGCTCTTCAGTTTTTCTGTTTCCTCAAGTCTCAGTGCTATCCTAT
TACAACATGGCTGATCATTAATTAGAAG

5.4.3 FABP7 mRNA sequence

CAGCCTCTTGGAAAGAGGGACACTGGAGGGGTGTGTTTGCAATTTAAATCACTGGATTTTTGCCCACCCTC
TTTCCAAATAAGAAGGCAGGAGCTGCTTGCTGAGGTGTAAAGGGTCTTCTGAGCTGCAGTGGCAATTAGA
CCAGAAGATCCCCGCTCCTGTCTCTAAAGAGGGGAAAGGGCAAGGATGGTGGAGGCTTTCTGTGCTACCT
GGAAGCTGACCAACAGTCAGAACTTTGATGAGTACATGAAGGCTCTAGGCGTGGGCTTTGCCACTAGGCA
GGTGGGAAATGTGACCAAACCAACGGTAATTATCAGTCAAGAAGGAGACAAAGTGGTCATCAGGACTCTC
AGCACATTCAAGAACACGGAGATTAGTTTCCAGCTGGGAGAAGAGTTTGATGAAACCACTGCAGATGATA
GAAACTGTAAGTCTGTTGTTAGCCTGGATGGAGACAAACTTGTTCACATACAGAAATGGGATGGCAAAGA
AACAAATTTTGTAAGAGAAATTAAGGATGGCAAAATGGTTATGACCCTTACTTTTGGTGATGTGGTTGCTG
TTCGCCACTATGAGAAGGCATAAAAATGTTCCTGGTCGGGGCTTGGAAGAGCTCTTCAGTTTTTCTGTTTCC
TCAAGTCTCAGTGCTATCCTATTACAACATGGCTGATCATTAATTAGAAGGTTATCCTTGGTGTGGAGGTGG
AAAATGGTGATTTAAAAACTTGTTACTCCAAGCAACTTGCCCAATTTTAATCTGAAAATTTATCATGTTTTAT
AATTTGAATTAAAGTTTTGTCCCCCCCCCCCTTTTTTTTATAAACAAGTGAATACATTTTATAATTTCTTTTGG
AATGTAAATCAAATTTGAATAAAAATCTTACACGTG

5.4.4 Validation of vectors for transfection

Figure 5.1 shows the products after agarose gel electrophoresis. The undigested vector ran at approximately 3.8kB, the single digest with SalI product over 5kB and there were 2 products of the double digest with SalI and XhoI one at about 4kB and the other at about 0.9kB.

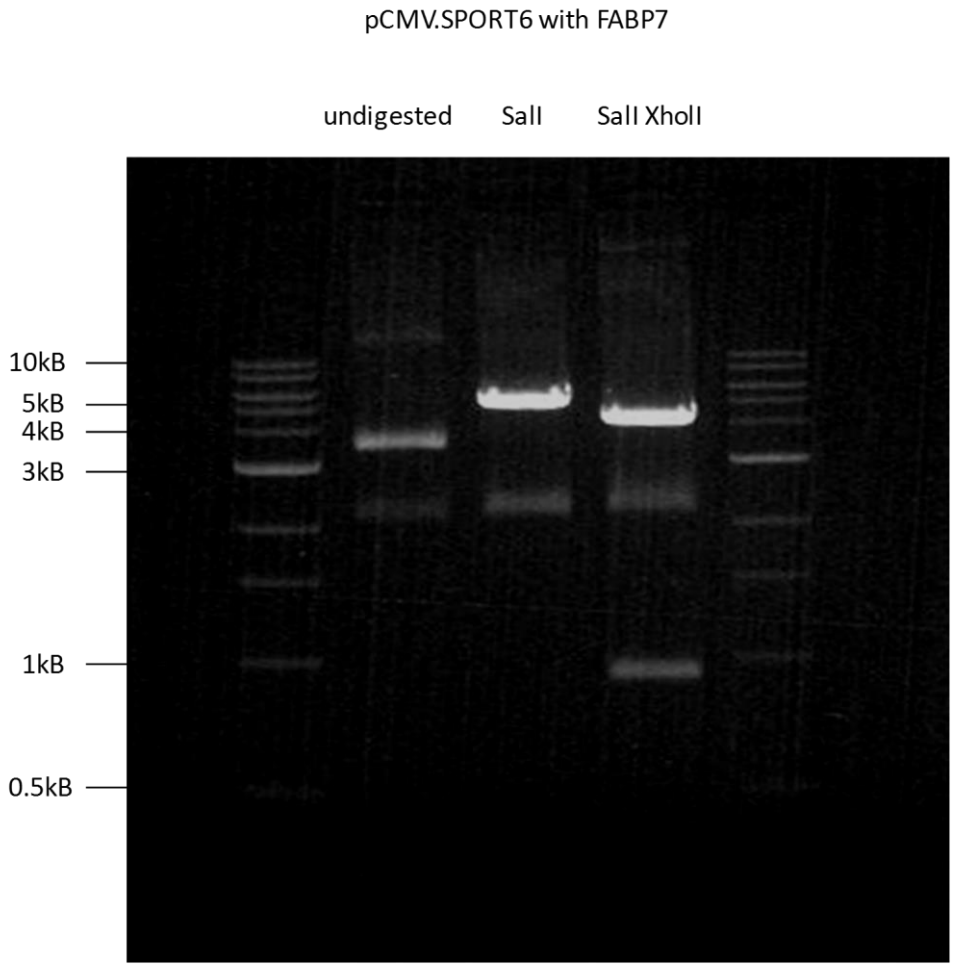


Figure 5.1 Restriction enzyme digest of pCMV.SPORT6 containing FABP7 insert
The vector was subjected to a single digest with SalI and double digest with SalI and XhoI. The undigested vector was also run alongside on the gel; this resulted in a band at around 3.8kB. The single digest resulted in a product of over 5kB and the double gave 2 products of sizes of about 4kB and 0.9kB.

The single digest resulted in a band at over 5kB for the vector with FABP7 and a band between 4kB and 5kB for the empty vector. The double digest resulted in 2 products for the FABP7 vector, one at around 0.9kB the other between 4 and 5kB. There was one visible product for the double

digest of the Empty vector at around 4kB. The bands of both the FABP7 and empty pCMV.SPORT6 digests were of the expected sizes from the restriction sites on the vector map.

An empty vector was synthesised for use as a negative control. From figure 5.2 both the restriction enzyme digests for the FABP7 pCMV.SPORT6 vector and the empty pCMV.SPORT6 vector resulted in the expected size bands. In the first digest in figure 5.1 there are 'ghost' bands present; these are likely to be denatured or nicked plasmid. Denatured or damaged plasmid DNA may have resulted in detrimental effects during transfection; to minimise this each new plasmid preparation was subjected to gel electrophoresis to ensure 'ghost' bands were minimal as in figure 5.2

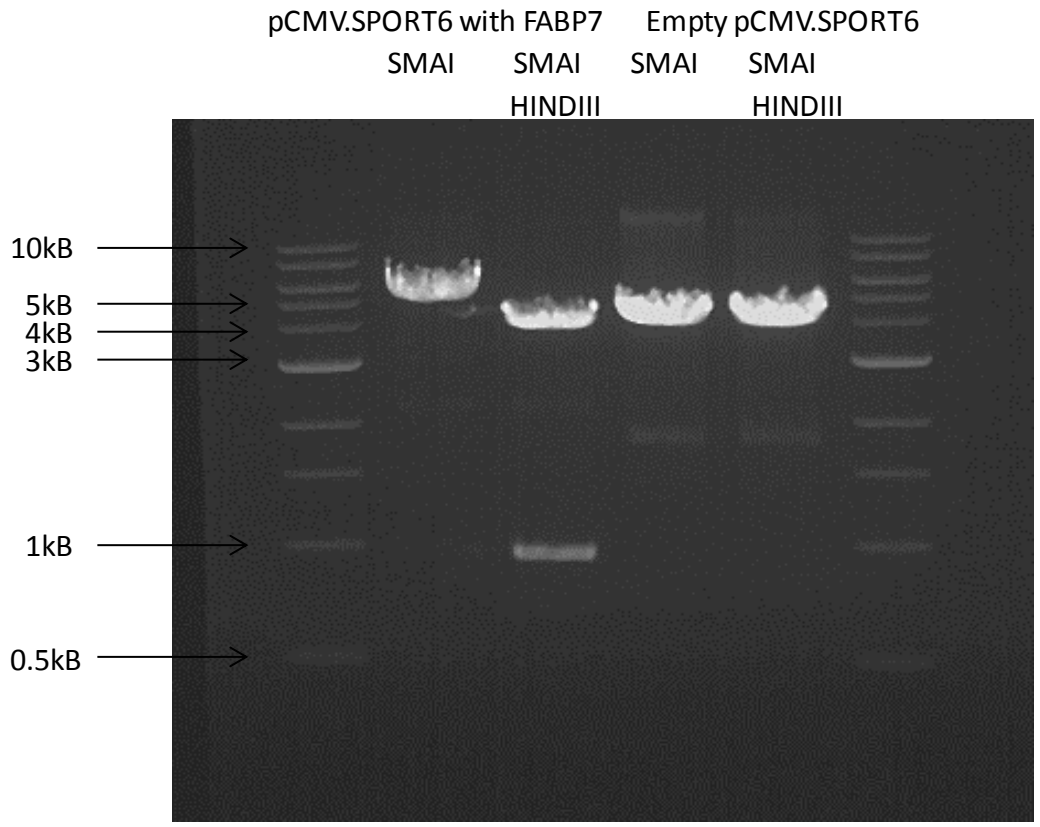


Figure 5.2 Restriction enzyme digest of pCMV.SPORT6 with FABP7 and Empty pCMV.SPORT6 vector to verify empty vector generation.
The vectors were subjected to a single digest with SmaI and double digest with SmaI and HindIII. For the FABP7 vector the single digest resulted in a product of over 5kB and the double gave 2 products of sizes of about 4kB and 0.9kB. For the single and double digest of the empty vector a band for each was seen at around 4kB.

5.4.5 Over expression of FABP7 in BT-20 cells

The BT-20 cell line was transfected with the pCMV.SPORT6 vector containing the FABP7 gene; RNA and protein were collected at 24, 48 and 72 hours post transfection. For use as controls BT-20 cells remaining un-transfected, treated with FugeneHD alone and transfected with a control pCMV.SPORT6 vector. Results are shown in figure 5.3, both protein and mRNA (approx 100 fold) were significantly increased after 24 hours ($p=0.0041$). The highest amount of protein and mRNA was seen at 48 hours post transfection with around a 400-430 fold increase in mRNA relative to untreated BT-20 cells ($p=0.001$). At 72 hours there was a drop in FABP7 mRNA to around 200 fold, compared to 48 hours, though this was still significant ($p=0.001$) compared to non-transfected cells. FABP7 protein levels were not notably different between 48 and 72 hours post transfection. There were no significant changes in FABP7 mRNA at any time point for the controls and FABP7 protein was not detected in the controls with western blotting.

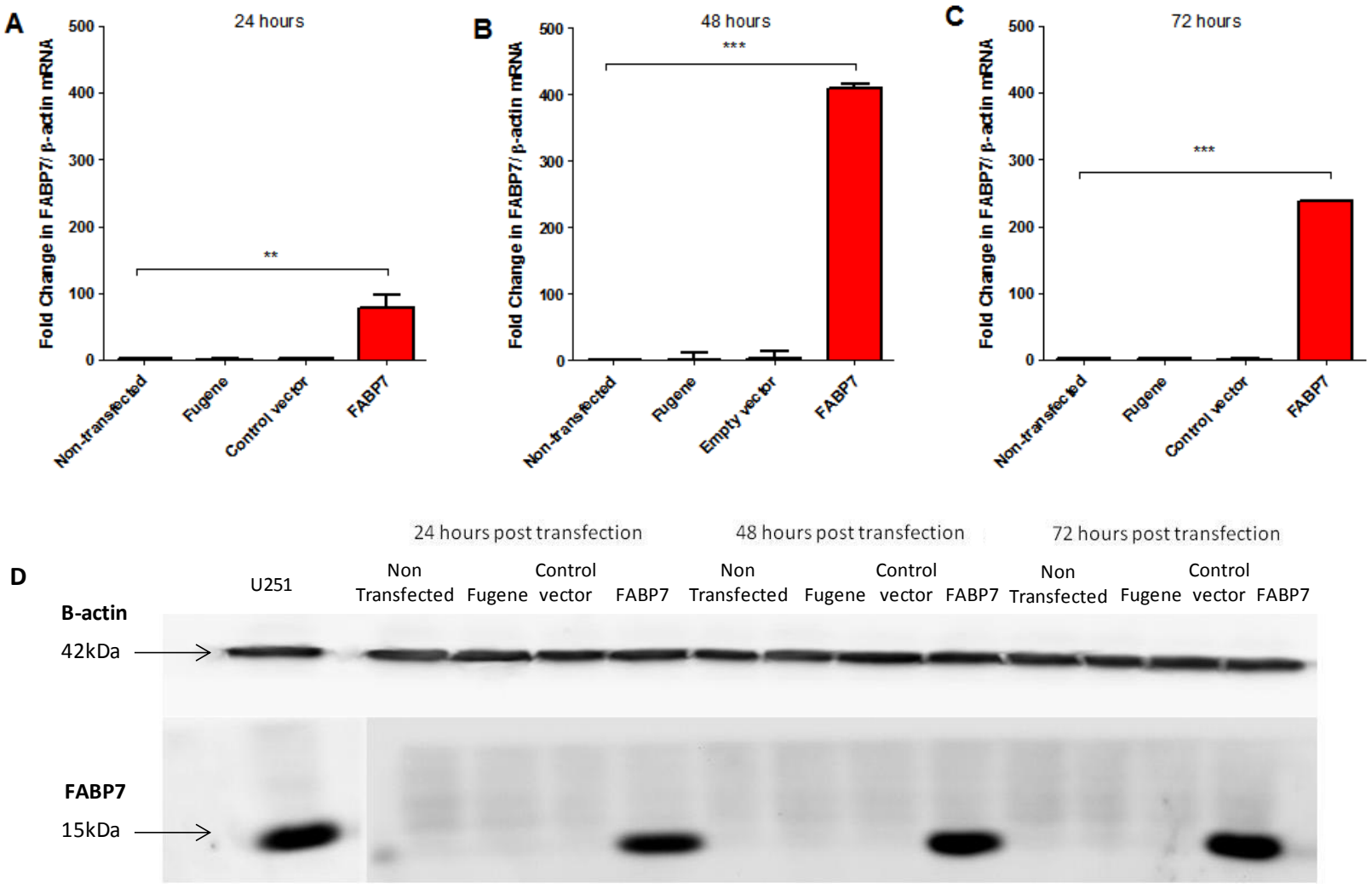


Figure 5.3 Over-expression of FABP7 in BT-20 cells

BT-20 cells were transfected with control or FABP7 containing pCMV.SPORT6 vector or left un-transfected or treated with Fugene alone. qPCR results are presented as fold change relative to non-transfected cells. FABP7 mRNA was increased in FABP7 transfected BT-20 cells at all time points. A) 24 hours approx 100 fold **(p=0.0041). B) 48 hours 400-430 fold ***(p=0.001). C) 72 hours approx240 fold ***(p=0.001). A western blot was carried out (D) on each condition at each time point; FABP7 protein detected in FABP7 over-expressing cells at all time points with maximal protein at 48-72 hours. Results are representative of 3 independent experiments with mean ±SEM as appropriate. The U251 FABP7 positive control was imaged separately.

5.4.6 FABP7 over-expression leads to a reduction in cell death relative to control vector transfection

Dead and live cells were counted post transfection as in figure 5.4 A) 24 hours, B) 48 hours and C) 72 hours. Results are expressed as percentage cell death and D) total cell number of transfected BT-20 cells across all time points. At 24 hours there were significant increases in percentage cell death with the control vector (15% p=0.002) and with FABP7 (11% p=0.007) compared to non-transfected cells. There was a significant decrease in percentage cell death in FABP7 transfected cells compared to control vector transfected cells (p=0.028). At 48 hours the only significant change in percentage death was between the untreated cells and the control vector transfection (11.8% p=0.0462). At 72 hours percentage cell death was again significantly increased between the untreated cells and the control vector and in FABP7 transfected cells to 24% (p=0.046) and 17.8% (p=0.049) respectively. There was also a significant difference between percentage cell death of control vector and FABP7 transfected cells (p=0.0307). Fugene HD treatment did not significantly alter percentage cell death at any time point. The treatments with Fugene HD, control vector and FABP7 transfection resulted in fewer total cells than the untreated cells across all time points.

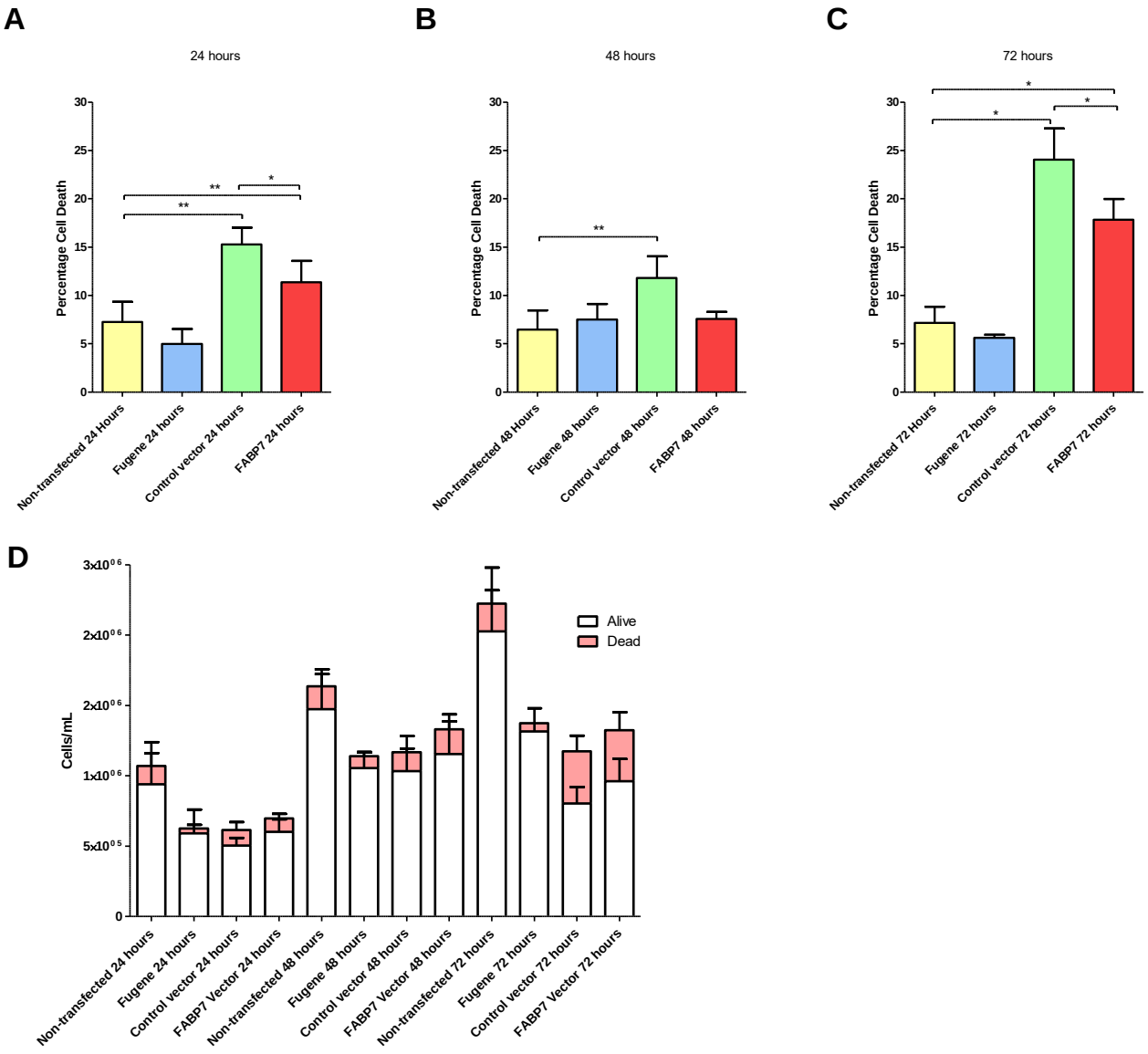


Figure 5.4 Percentage cell death after transfection

Live cells were trypsinized and counted with dead cells using Trypan blue, post transfection, after A) 24 hours [significant differences: no treatment vs control vector **(p=0.002); no treatment vs FABP7 vector **(p=0.007); control vector vs FABP7 vector *(p=0.028)], B) 48 hours and [significant differences: untreated vs control vector **(p=0.0462)] C) 72 hours [significant differences: untreated vs control vector *(p=0.046); untreated vs FABP7 vector *(p=0.049); control vector vs FABP7 vector *(p=0.0307)]. Results are expressed as percentage cell death. D) Total cell number of transfected BT-20 cells across all time points are included to gauge changes in cell growth. Results shown represent 3 independent experiments carried out in triplicate with mean ±SEM.

5.4.7 FABP7 over-expression lead to increased cell viability compared to control vector cells assessed with an MTT assay

An MTT assay was used to investigate cell viability post transfection. The MTT assay results are in figure 5.5; there was no significant change in mitochondrial activity/cell viability between the non-transfected cells and the FABP7 transfected cells. There was a significant decrease in cell viability/mitochondrial activity in the control vector transfected cells compared to both non-transfected (p=0.0039) and FABP7 transfected BT-20 cells (p=0.0002).

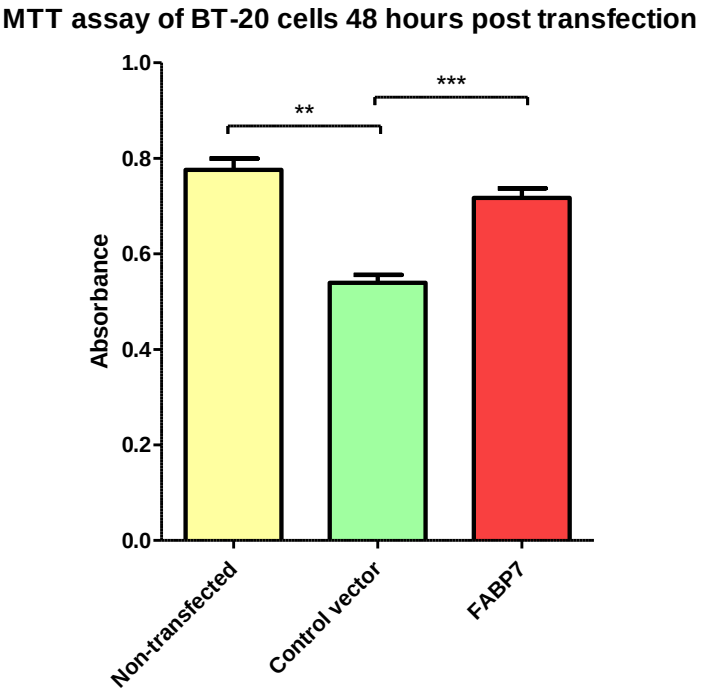


Figure 5.5 MTT assay of transfected BT-20 cells

BT-20 cells were transfected and the mitochondrial/cell viability measured with an MTT assay. There was no significant change in mitochondrial activity/cell viability between the untreated cells and the FABP7 transfected cells. There was a decrease in cell viability/mitochondrial activity in the control vector transfected cells compared to the non-transfected cells **(p=0.0039) and the FABP7 transfected cells **(p=0.0002). Results shown represent the mean of 3 independent experiments that were carried out in quintuplicate ±SEM.

5.4.8 FABP7 transfected BT-20 cells are more resistant to death by FAs than control vector transfected cells

Considering that there were changes in cell death and viability as a result of the transfection, another question to answer was whether transfection with FABP7 conferred more or less sensitivity to cell death by FAs. The results are shown in figure 5.6. A control experiment was carried out in conjunction to ensure serum starvation did not impact significantly on cell death post transfection; the percentage cell deaths were very similar to the original transfection cell count experiments as in figure 5.4. There was a significant increase in percentage cell death with 50µM of palmitate in the control vector transfection relative to non-transfected cells (p=0.0006) and there was a trend for the FABP7 transfected cells to have lower percentage cell death compared to the control vector but this was not significant. A dose of 200µM palmitate resulted in a significant increase in percentage cell death in the control vector (55.6%) compared to both the non-transfected cells (34.1% p=0.0213) and the FABP7 transfected cells (41.1% p=0.046) though there was no difference between the non- and FABP7 transfected cells. Oleate doses used were 50µM and 100µM; a significant increase in cell death was noted for 50µM in the control vector transfected cell (31.9%) compared to both the non- (9.6%, p=0.010) and FABP7 (21.1%, p=0.011) transfected cells. The percentage cell death in the FABP7 transfected cells was higher than the cells with only oleate but this did not reach statistical significance. With 100µM oleate the same trend was present with 42.5% cell death in the control vector transfected cell compared to 15.6% (p=0.0014) for non-transfected and 14.3% in FABP7 over-expressed cells (p=0.0027). Doses of AA used were 10µM and 50µM; the only significant difference in percentage cell death was for 10µM between the non-(12.4%) and control (22.9%) vector transfected cells (p=0.0049); however there was a trend for the percentage cell death in FABP7 over-expressing cells to be lower than both the non- and control vector transfected cells with 50µM AA. DHA was used at doses of 10µM and 50µM; there was a significant reduction in cell death between the control vector and FABP7 transfection with both doses (10µM: 24.6% vs 15.4% p=0.0473; 50µM: 25.4% vs

15.9% p=0.0156); there was also a trend FABP7 transfected cells to have a lower percentage cell death than the non-transfected BT-20 cells.

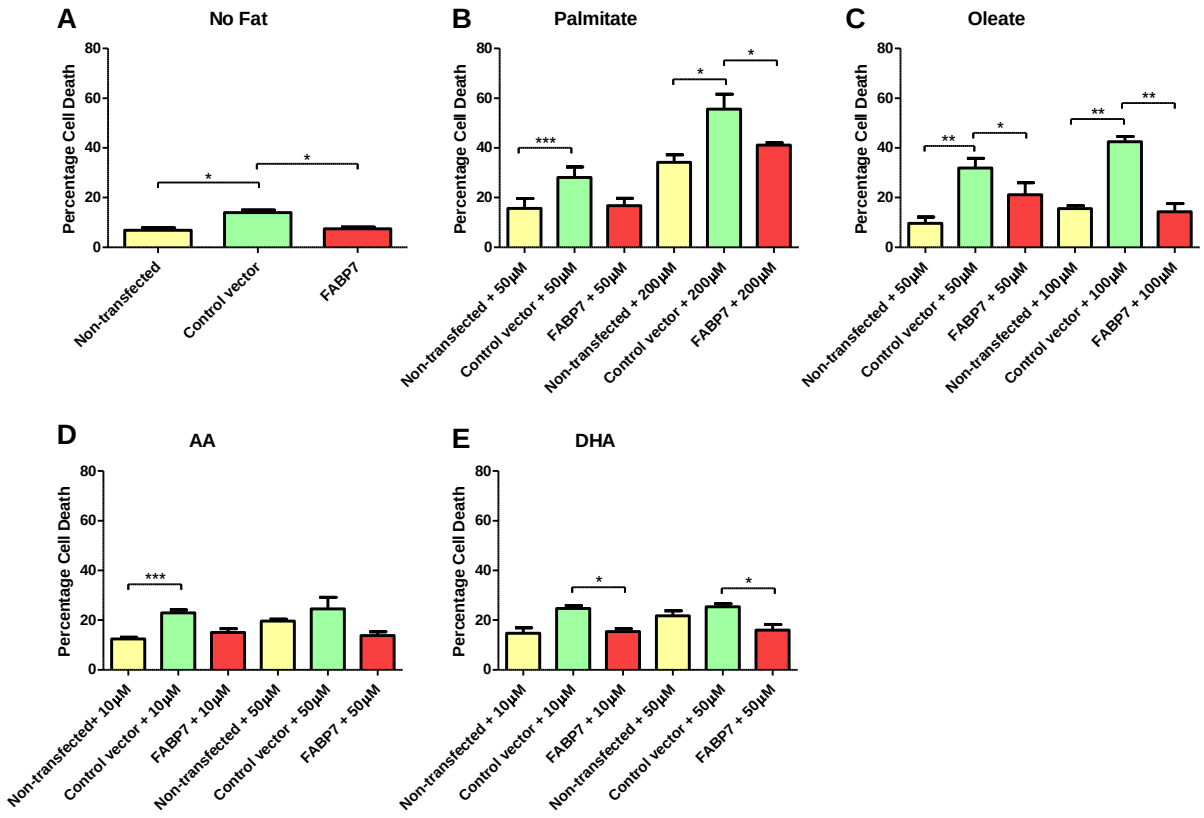


Figure 5.6 Treatments of FA on transfected BT-20 cells

A) No fat was used as a control to ensure the lack of serum did not impact on cell death; the percentage cell deaths were very similar to the original transfection cell count experiments (figure 5.4). B) There was a significant increase in percentage cell death with 50µM palmitate in the control vector transfection relative to untreated cells **(p=0.0006); 200µM palmitate caused a significant increase in percentage cell death in the control vector (55.6%) compared to both the non-transfected cells *(34.1% p=0.0213) and the FABP7 transfected cells *(41.1% p= 0.046). C) An increase in cell death was noted for 50µM oleate in the control vector transfected cell (31.9%) compared to both the non- **(9.6%, p=0.010) and FABP7 *(21.1%, p=0.011) transfected cells. With 100µM oleate the same trend was present with 42.5% cell death in the control vector transfected cell compared to 15.6% **(p=0.0014) for untreated cells and 14.3% in FABP7 over-expressed cells **(p=0.0027). D) The only significant difference in percentage cell death after AA treatment was for 10µM between the non-(12.4%) and control (22.9%) vector transfected cells ***(p=0.0049). E) There was a significant reduction in cell death between the control vector and FABP7 transfection at both doses (10µM: 24.6% vs 15.4% *p=0.0473; 50µM: 25.4% vs 15.9% *p=0.0156). Results shown represent the mean of 3 independent experiments carried out in triplicate with ±SEM. A one-way ANOVA was used with a Bonferroni post-hoc test; a student's paired t-test was used to determine any specific differences.

5.4.9 Transfection with FABP7 in BT-20 cells did not alter FA accumulation

Considering that FABP7 binds the FAs used in this study, oil red O staining was carried out on transfected cells that had been treated with each of the FA to see if there were any changes in FA accumulation or lipid droplet formation. The stained cells were assessed visually with representative results displayed in figure 5.7; there was no noticeable change in FA accumulation as a result of the transfection. Oleate was the only FA that accumulated in the BT-20 cells.

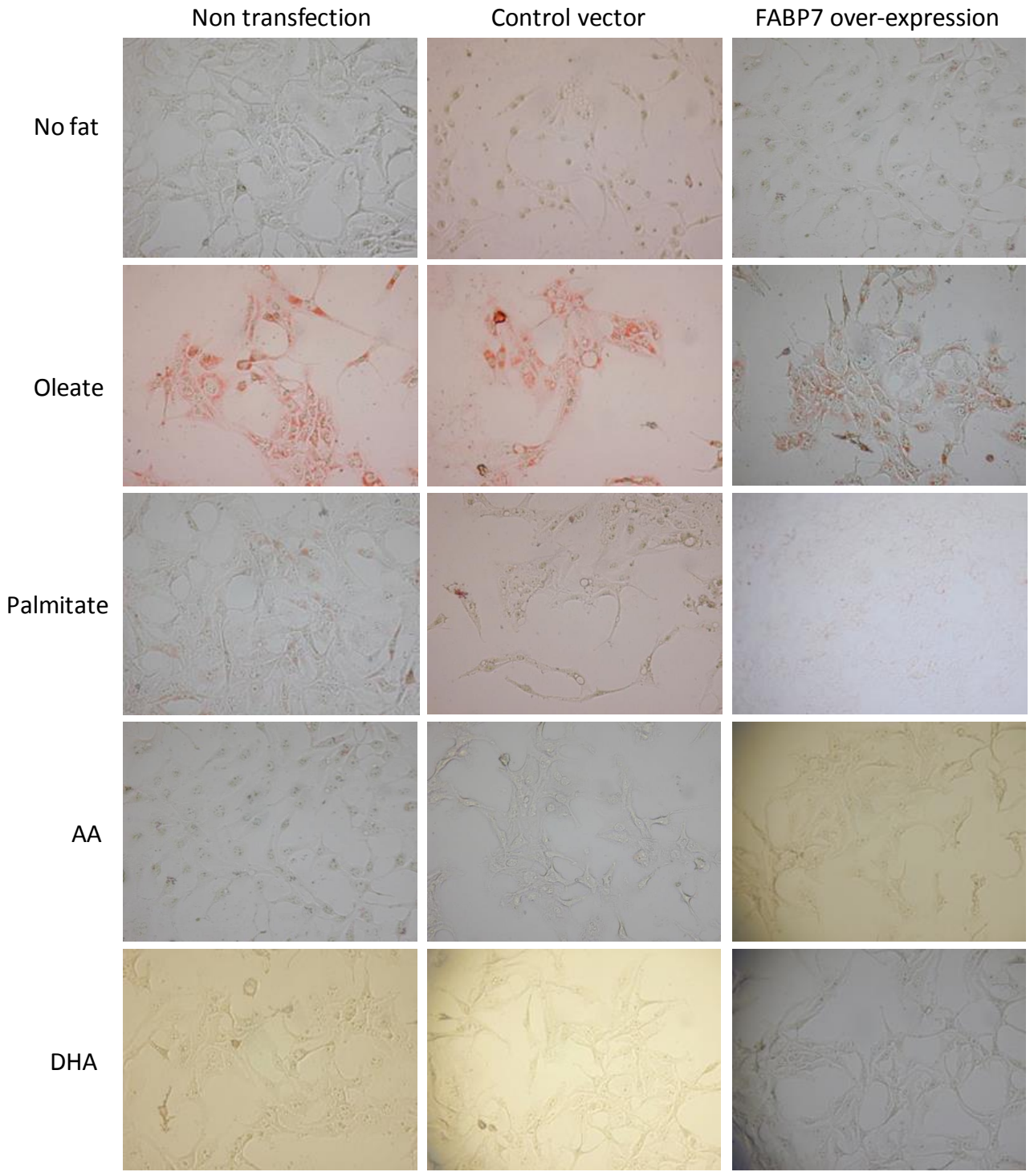


Figure 5.7 Oil red O staining of FA uptake in transfected BT-20 cells

Control and transfected BT-20 cells were treated with either oleate, palmitate, AA or DHA and then stained with oil red O; staining was assessed by eye using a microscope. Oleate resulted in FA accumulation in the BT-20 cells leading to strong staining. AA and DHA treatment did not give any oil red O positivity. There was no visible difference in FA accumulation as a result of transfection with FABP7 compared to both control vector transfected and untreated cells. Results shown are representative of 3 independent experiments.

5.4.10 FABP7 over-expression did not confer resistance to apoptosis induced by STRP

In addition to treatment with fat, BT-20 cells were treated with STRP post transfection to investigate whether FABP7 reduced apoptosis by STRP. Non-transfected cells were treated with and without STRP to show that STRP induced death; there was 14.6% cell death in untreated cells compared to 39.3% with the addition of STRP (p=0.0001). As previously, there was a lower percentage cell death in untreated cells (14.6% p= 0.0004) and FABP7 transfected cells (14.6% P=0.0002) compared to the cells transfected with the control vector (23.5%). There was a significant increase in cell death in the control vector transfected cells treated with STRP (45.6%) relative to the non-transfected cells with STRP (39.3% p=0.0334) however there was no significant change in percentage cell death for the FABP7 transfected cells treated with STRP (43.5%) relative to the non-transfected cells with STRP (39.3%). Results are in figure 5.8

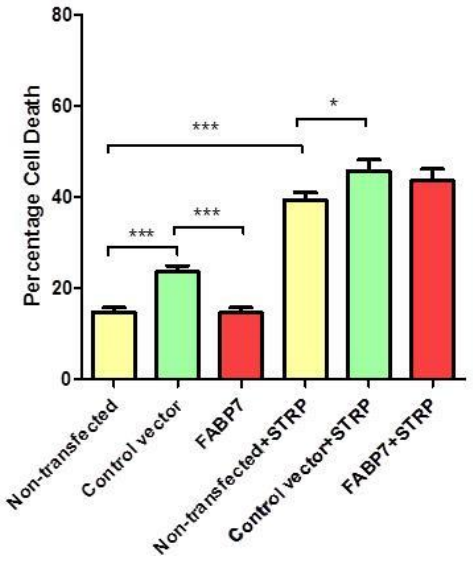


Figure 5.8 Transfected BT-20 cells treated with STRP

Transfected and control BT-20 cells were treated with STRP to look for changes in sensitivity to cell death. STRP induced 39.2% in non-transfected cells compared to 14.6% without ***(p=0.0001). Control vector transfection (23.6%) resulted in higher percentage cell death than non-transfected ***(14.6% p=0.0004) and FABP7 transfected cells ***(14.6% p=0.0002). STRP treatment resulted in a higher percentage cell death in control vector transfected cells (45.6%) than cells treated with STRP alone *(39.3 p=0.0334). A one-way ANOVA was used with a Bonferroni post-hoc test; a student's paired t-test was used to confirm any differences. Results shown represent the mean of 3 independent experiments carried out in triplicate ±SEM

5.4.11 There was no significant difference in the ratio of expression of bcl-x splice variants in BT-20 cells transfected with FABP7

Changes in *BCL-X* splice variant expression were assessed by PCR and band densitometry 24, 48 and 72 hours post transfection. Results are in figure 5.9; A is an example of bcl-x bands on a gel at the 24 hour time point, B is the band densitometry ratio for each treatment at each time point and represents the mean of three independent experiments ±SEM. The ratio of the two bands was calculated by dividing the intensity of the 350bp anti-apoptotic band by the 160bp pro-apoptotic band so that the larger the ratio the less apoptotic potential. There were no significant changes in *BCL-X* expression as a result of transfection with either the control vector or FABP7 at any time point. The greatest change was at 48 hours where the ratio for FABP7 transfected cells was 2.06 compared to the untreated and control vector transfected cells which both had a ratio of around 2.5.

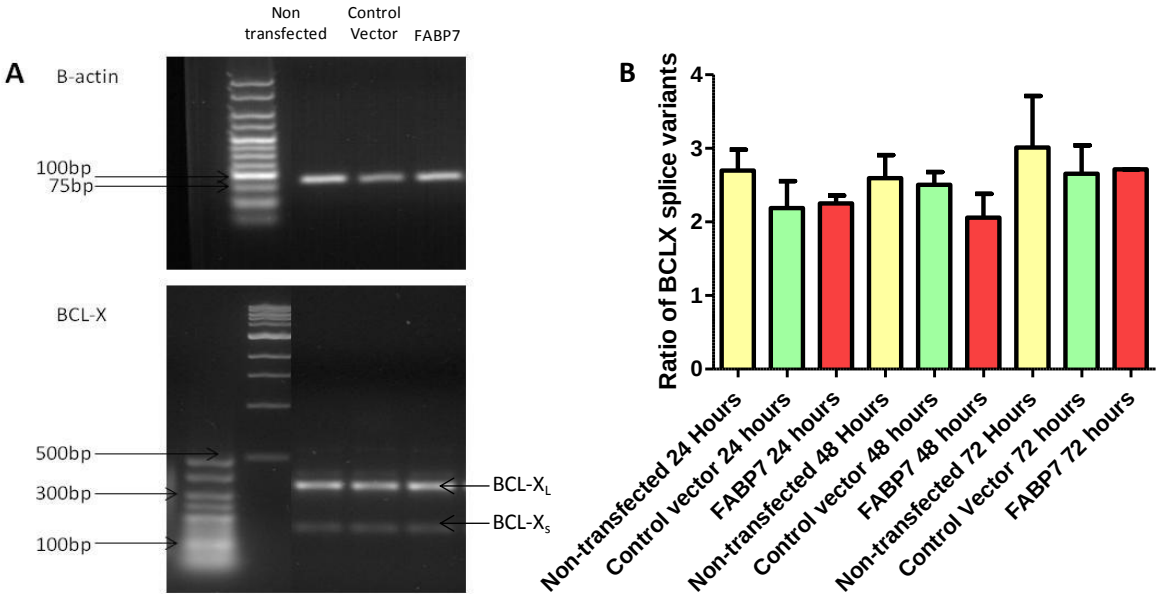


Figure 5.9 Expression of BCL-X mRNA does not change significantly as a result of transfection with FABP7

An example of the PCR BCL-X gel at the 24 hour time point. B) Band densitometry of BCL-X splice variants; a larger ratio indicates less apoptotic potential. There were no significant differences in bcl-x mRNA expression at anytime point for any treatment. A one-way ANOVA was used with a Bonferroni post-hoc test; a student's paired t-test was used to confirm any differences. Results represent the mean of 3 independent experiments carried out in triplicate ±SEM.

5.4.12 FABP7 transfected cells tended to have higher expression of the anti-apoptotic splice variant ΔEx3 survivin

BT-20 cells were transfected with a control vector or FABP7 and RNA was extracted after 24, 48 and 72 hours. Standard PCR and band densitometry was initially carried out on survivin splice variants; there was a trend noted that FABP7 transfected cells tended to have higher expression of anti-apoptotic splice variants (figure 5.10)

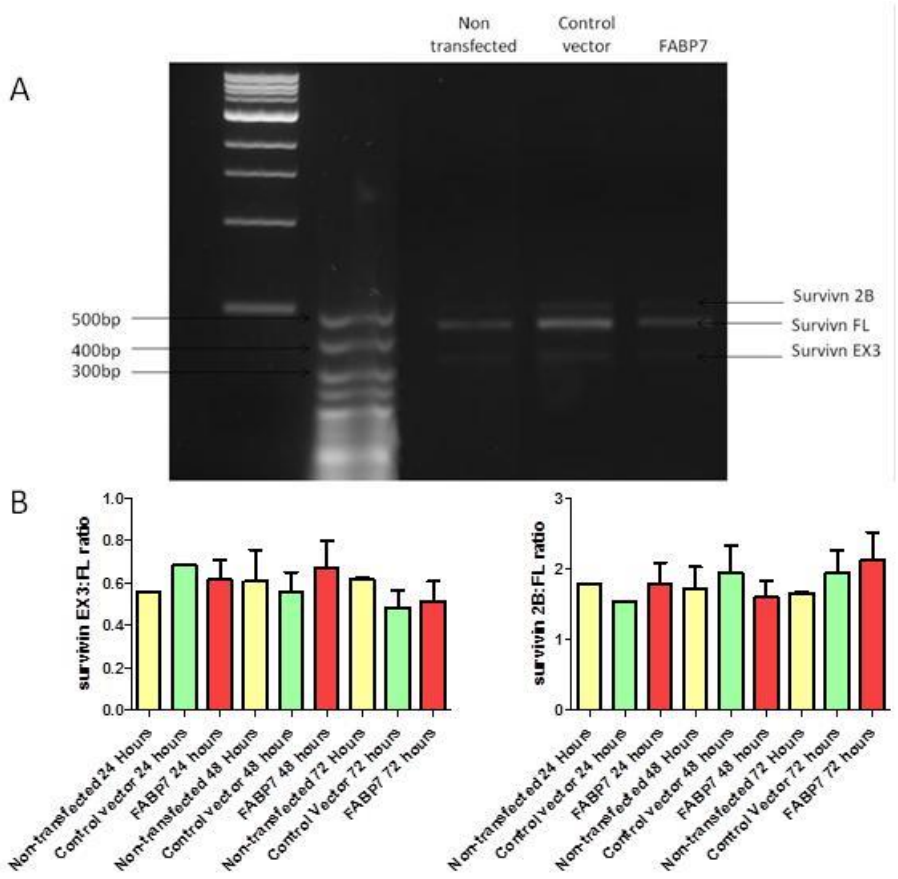


Figure 5.10 Standard PCR and densitometry analysis of survivin splice variant expression

This figure shows a gel representative of survivin splice variant expression in BT-20 cells after transfection (A). Densitometry was carried out to give the expression ratio of survivin EX3 and 2B (B). There were trends for FABP7 over-expressing cells to have more anti-apoptotic potential; to confirm these trends qPCR was carried out.

To investigate these trends further qPCR was carried out, the results are in figure 5.11. There were no-significant changes in total (A) or survivin splice variant expression (B&C) or the ratios (D&E) at any time point. However there were consistent trends noted. Full length survivin (survivin FL) expression tended to be higher in the FABP7 over-expressing cells compared to the control vector transfected and un-transfected cells; survivin FL expression was at its highest at 48 hours where there was a 1.88 fold increase relative to un-transfected cells. The control vector transfected cells had lower expression of survivin FL in relation to untreated cells at 24 and 48 hours (both around 0.67 fold). The same trends were present for the expression of survivin ΔEx3;

there was a 1.59 fold increase of survivin ΔEx3 in FABP7 transfected cells at 48 hours. Survivin ΔEx3 in control vector transfected cells did not differ from the un-transfected cells until 72 hours where it fell to 0.56 fold. In contrast to this there were not any notable changes in survivin 2B as a result of control vector or FABP7 transfection until 72 hours where there was a reduction for both, 0.56 fold and 0.77 fold respectively. To investigate changes in apoptotic or survival potential the ratio of the splice variant relative to survivin FL was calculated; the smaller the ratio the less the anti-apoptotic properties and vice versa. The survivin ΔEx3 to survivin FL ratio calculation showed that there was no change in anti-apoptotic properties as a result of control vector transfection compared to un-transfected cells; the ratios remained between 1.3 and 1.5. There were trends for the ratio of the splice isoforms in FABP7 over-expressing cells to be higher than both control and un-transfected cells; the highest ratio change was at 72 hours (ratio 2.3). The survivin 2B to survivin FL ratio changes did not change notably with the exception at 24 hours where both the ratio of control vector and FABP7 were lower than untreated cells (1.5), 0.9 and 1.1 respectively. The ratios for 48 and 72 hours were all between 1.0 and 1.3.

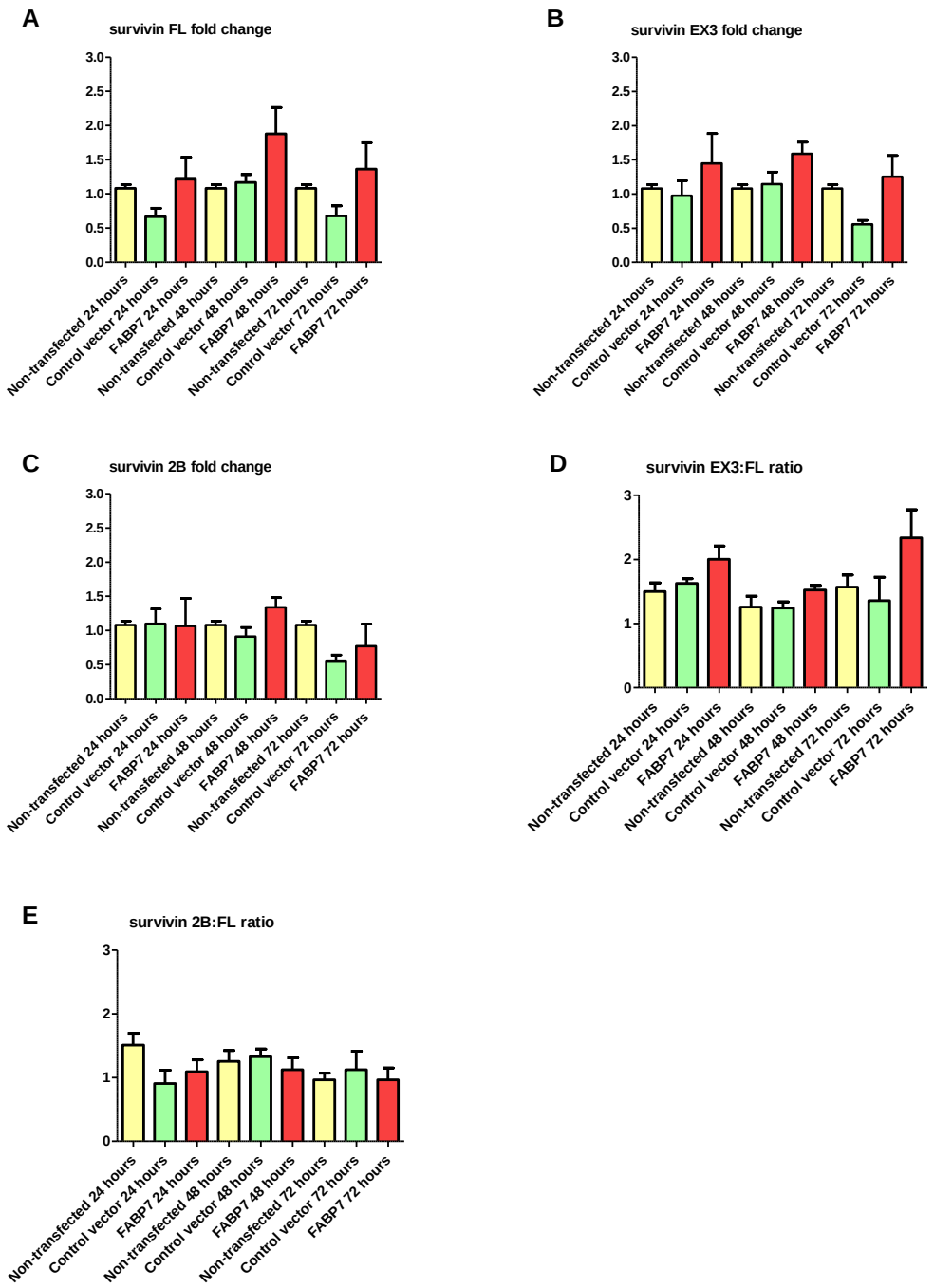


Figure 5.11 Survivin FL and ΔEx3 increase as a consequence of FABP7 over-expression

A) Fold change in survivin FL mRNA. B) Fold change in survivin ΔEx3 mRNA. C) Fold change in survivin 2B mRNA. D) The ratio of survivin ΔEx3 to survivin FL. D) The ratio of survivin 2B to survivin FL. There were no statistically significant changes in expression or ratio of the splice variants, though there was a trend for FABP7 over-expressing cells to have a higher expression of survivin FL and survivin ΔEx3 compared to both non- and control vector transfection. Results shown represent the mean of 3 independent experiments carried out in triplicate ±SEM. A one-way ANOVA was used with a Bonferroni post-hoc test; a student's paired t-test was used to confirm any differences.

5.4.13 FABP7 transfection did not alter the ratio of caspase 9 splice variants significantly, but there were changes in overall splice variant expression

Standard PCR and band densitometry was initially carried out for caspase 9 splice variants in transfected BT-20 cells. Changes were inconsistent over time as seen in figure 5.12.

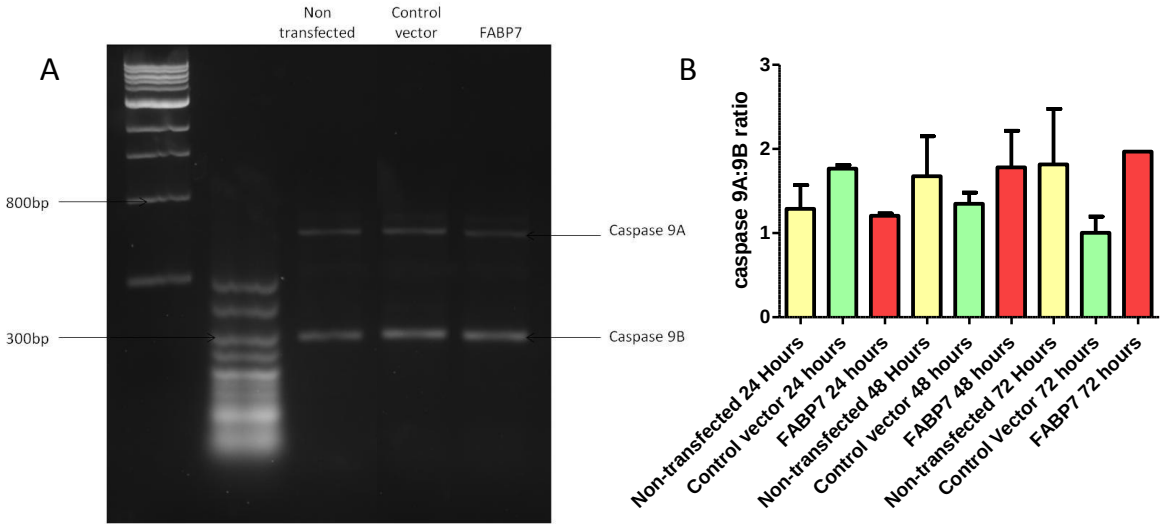


Figure 5.12 Standard PCR and band densitometry analysis of caspase splice variant expression
This figure shows a gel representative of survivin splice variant expression in BT-20 cells after transfection (A). Densitometry was carried out to give the expression ratio caspase splice variant expression (B). The trends over time were inconsistent so qPCR was carried out to further investigate changes in expression.

To further investigate changes in caspase 9 splice variant expression qPCR was carried out (figure 5.13). There were no statistically significant changes in caspase 9A expression as a result of transfection with either the control or FABP7 vector transfection. Caspase 9A fold expression in control vector transfected cells were consistently lower relative to non-transfected cells, 0.44, 0.54 and 0.64 at 24, 48 and 72 hours respectively. In contrast the fold change in expression, relative to non-transfected cells, did not alter in FABP7 transfected cells until after 72 hours where it fell to 0.45. At 24 hours there was a reduction in caspase 9B expression (0.49 fold). Forty-eight hours post transfection levels of caspase 9B were similar across treatments. Caspase

9B fold change decreased for both transfected cells sets at 72 hours to 0.42 (control vector) and 0.49 (FABP7). To confirm any differences the ratio of expression of the 2 splice variants was calculated. There was little difference in the ratios for each treatment and across each time point. The largest difference was a reduction in the ratio in FABP7 transfected cells to 0.96 compared to 1.3 for both non- and control vector transfected cells after 24 hours.

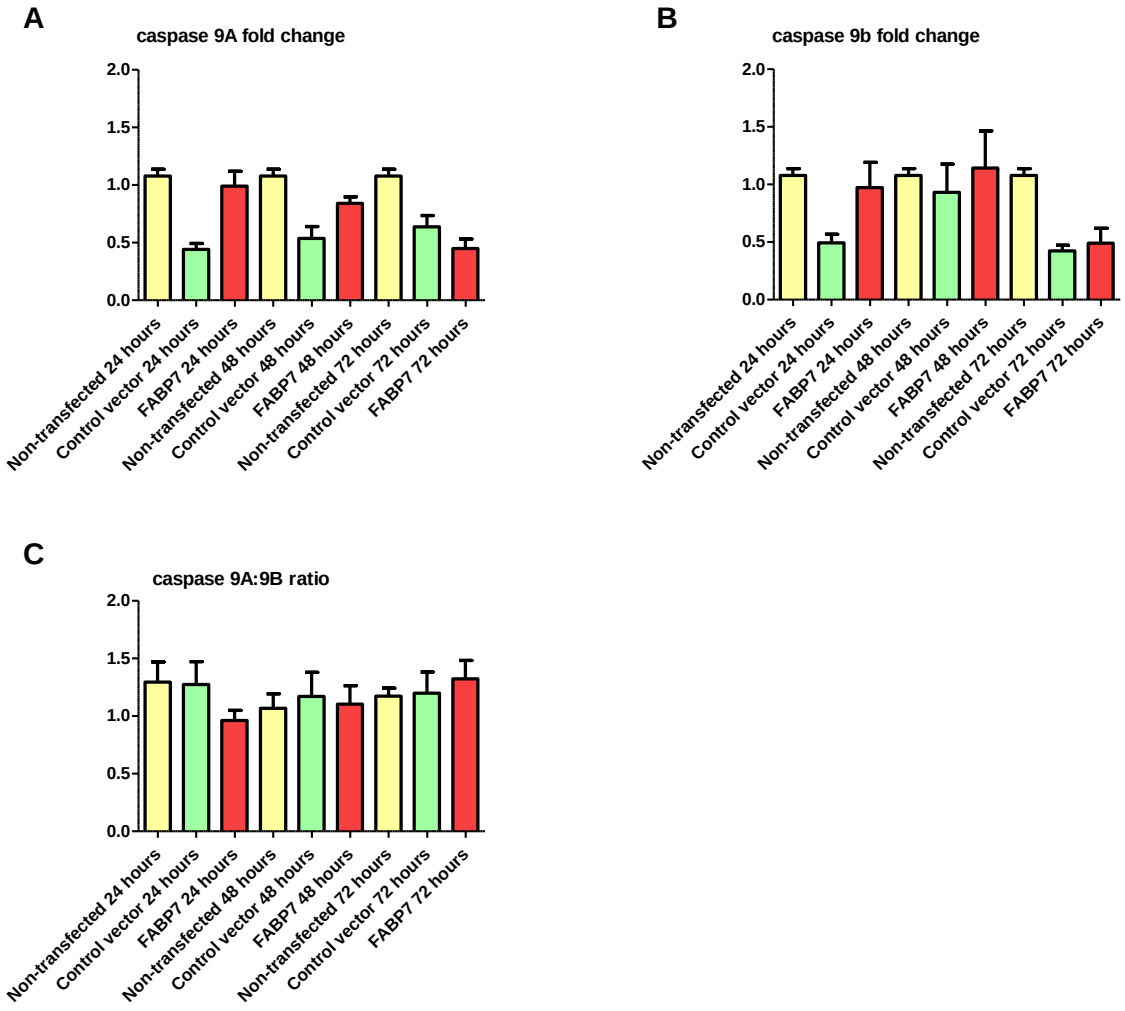


Figure 5.13 Changes in expression of caspase 9 splice variants after transfection

A) Fold change in caspase 9A expression relative to untransfected cells. B) Fold change in caspase 9B expression relative to untransfected cells. C) The ratio of caspase 9A to 9B expression. There were no significant differences in overall caspase 9 splice variant expression or ratio of splice variant expression. Results shown represent the mean of 3 independent experiments carried out in triplicate ±SEM. A one-way ANOVA was used with a Bonferroni post-hoc test; a student's paired t-test was used to confirm any differences.

5.5 Discussion

Transfection was successful with no increases in FABP7 mRNA or protein as a result of FugeneHD alone or control vector transfection. FABP7 mRNA was maximal after 48 hours and then dropped slightly at 72 hours. Protein was present after 24 hours with bands being comparable at 48 and 72 hours. This is as predicted as it would take time for cells to produce the protein after transfection and as the transfection is only transient levels of both protein and mRNA would be expected to decrease beyond 72 hours.

5.5.1 FABP7 over-expression reduced cell death and increased cell viability relative to control vector transfected cells

Live and dead cell counting after trypan blue staining has been shown to give comparable results to other methods such as flow cytometry and PARP cleavage assessment by western blotting (Thomas *et al.*, 2010; Fowler *et al.*, 2000; Gill *et al.*, 1997). However counting dead cells with trypan blue can have limitations due to disintegration of dead cells. The results showed that percentage cell death was minimal at 24 and 48 hours but at 72 hours percentage cell death in both transfected sets of cells increased dramatically indicating that the transfection was beginning to have adverse effects on cell viability. The cell counts also showed that the control vector transfected cells consistently had higher percentage cell death compared to both the untreated cells and the FABP7 transfected cells. This is unlikely to be a result of experimental error, for instance due to seeding densities; seeding densities were the same across all wells verified by the total cell count results. In addition nicked DNA assessed by restriction enzyme digest of plasmid, was minimal so unlikely to cause cell death. The differences noted in the total cell counts between the untreated versus the FugeneHD, control vector and FABP7 transfected conditions are more likely to reflect changes in growth although changes were not significant. The MTT assay results further confirmed statistically significant differences in cell viability

between the control vector and FABP7 transfected cells; however there was no difference in mitochondrial activity between FABP7 over-expression and non-transfected cells. One possible reason for this is that BT-20 cells may not cope well being transfected, perhaps the plasmid DNA activates certain pathways to cause death similarly to immune responses to viruses (Hornung & Latz, 2010; Atianand & Fitzgerald, 2013). FABP7 may activate pathways that are protective against death or deactivate apoptotic pathways; this theory has further evidence from the percentage cell death in FABP7 over-expressing cells reducing after 48 hours, relative to control vector transfected cells and cell death at 24 hours, when FABP7 expression is at its highest.

5.5.2 FABP7 over-expression conferred resistance to cell death by FAs relative to control vector transfected cells.

Transfected BT-20 cells were treated with 2 doses of palmitate, oleate, AA and DHA to investigate whether FABP7 was protective against cell death. Interestingly the control vector transfected cells consistently had a significantly higher percentage cell death compared to non-transfected cells for all FAs at both doses with the exception of 50µM AA and both doses of DHA. This could be due to the fact the cells are porous post-transfection so the FAs more readily enter the cells than usual. Moreover, cells over-expressing FABP7 had significantly lower cell death than control vector transfected cells for both doses of oleate, 200µM palmitate and for both doses of DHA. Again this is indicative that FABP7 protects against cell death and in this case death from FAs; however there were no differences in cell death between non-transfected BT-20 cells and those over-expressing FABP7. DHA has previously been shown to promote apoptosis and inhibit growth in breast cancer cell lines (Siddiqui *et al.*, 2005; Sun *et al.*, 2008). One study found that by knocking down FABP7, the MDA-MB-435 cell line became sensitive to the effects of DHA; growth inhibition became apparent (Liu *et al.*, 2012). In this study the approach was the opposite, FABP7 was over-expressed and the effect of DHA on cell death compared to controls was negated. This

was particularly apparent for 50μM DHA, although not significant, cell death in FABP7 transfected cells was 16% compared to 22% in non-transfected BT-20 cells. It was postulated that this effect could be a result of excess FABP7 sequestering DHA and thus limiting its availability or directing it to enzymes for degradation (Liu *et al.*, 2012). In contrast to this, another possibility is that FABP7 transports DHA to the nucleus to trigger survival and proliferation pathways (Liu *et al.*, 2012). This is in conjunction with work by Mita *et al* (2010); the role of FABP7 with DHA in growth, apoptosis and proliferation was studied in glioma cell lines. The findings of the study led them to come up with a suggested pathway (figure 1.13). FABP7 binds to AA or DHA depending on the relative abundance and the shuttles DHA into the nucleus which then binds to *PPAR-γ* resulting in down-regulation of promigratory genes or it binds to AA to transfer it to the COX2 pathway in the cytoplasm to activate promigratory genes downstream of the pathway (Mita *et al.*, 2010). The reasons for the different documented effects of DHA on proliferation and apoptosis are probably due to the cell lines being of different origin and having different gene expression profiles, in particular differing basal levels of FABP7. It is therefore probable that in the present study, FABP7 inhibits death induced by DHA by sequestering it and preventing gene activation. In light of the work by Mita *et al* (2010) it could be predicted that AA has the opposite effect however there were no significant changes in cell death with AA treatment in FABP7 transfected cells. Perhaps higher doses were required to see a difference in cell death with AA and a more marked effect with DHA. Oleate and palmitate have been demonstrated to have opposite effects on cell proliferation and apoptosis; therefore it was interesting that FABP7 transfected cells had a lower percentage cell death than control vector transfected cells with both oleate and palmitate treatments. Again, this could be due to FABP7 sequestering the FAs to prevent FAs causing cell death. The higher doses of oleate and palmitate did not reveal any significant differences between FABP7 transfected and non-transfected cells.

5.5.3 FABP7 over-expression did not alter FA accumulation

To ascertain if FABP7 had any impact on FA accumulation BT-20 cells were transfected and then treated with the FAs for staining with oil Red O. There was not any noticeable difference in FA accumulation as assessed by oil red O. Oleate was the only fatty acid that accumulated in the cytoplasm and therefore was strongly positive with oil red O staining however the degree of staining was not different between FABP7 over-expressing and control BT-20 cells. It is a possibility that AA and DHA do not accumulate in BT-20 cells or that the dose was not high enough to see an effect; higher doses would have been detrimental to the cells and perhaps there would not be any cells remaining to stain for after a higher dose of FA. In light of this FABP7 may not be involved with FA accumulation in breast cells and its role may be in solubilising and transporting FA when they have entered or are synthesised in the cells so that they are metabolised.

5.5.4 Over-expression of FABP7 did not alter sensitivity to apoptosis by STRP

To investigate whether FABP7 over-expression conferred resistance to apoptosis, BT-20 cells were transfected and then treated with STRP. STRP successfully induced apoptosis however only the control vector transfected cells were more susceptible to STRP compared to non-transfected cells treated with STRP. This increase in death could be viewed as the additive effect of the control vector transfection causing death with STRP rather than the cells being more sensitive particularly as the control vector transfection had a higher percentage cells death in the group without STRP treatment. It is not known how STRP induces apoptosis but it is thought that it does this through several different mechanisms (Weil *et al.*, 1996). It is possible that a difference in sensitivity was not seen because of this; FABP7 could confer resistance or sensitivity in apoptosis but only through 1 or 2 mechanisms and these would not be noticeable due to the multi-target effect of STRP. One way to overcome this is to use agents that induce apoptosis in a more specific manner

but clues would be needed as to which mechanisms FABP7 works with as the use of each agent would need to be justified. For these reasons different genes involved in apoptosis and survival were looked at for changes in expression as a result of FABP7 transfection.

5.5.5 Over-expression of FABP7 altered the expression of splice variants involved in apoptosis and survival

The genes in the present study were chosen for the reason that the splice variants could give information on changes in both anti- and pro-apoptotic properties at the same time. Standard PCR was carried out to initially look for changes and then qPCR was carried to confirm and changes. There were no significant changes in either of the bcl-x splice variants as a result of FABP7 over-expression. The bcl-x_L protein has been found to be the variant that is predominantly over-expressed in breast cancer and it is associated with high tumour grade (Olopade *et al.*, 1997). If FABP7 had been found to increase bcl-x_L or decrease bcl-x_s it could be indicative that it is of poor prognostic value. Interestingly there were changes in survivin expression. The survivin 2B splice variant changed the least in terms of overall expression and the ratio with survivin FL with transfection; therefore FABP7 expression did not lead to reduced anti-apoptotic properties. In contrast there were increases in survivin FL and survivin ΔEx3 in FABP7 over-expressing BT-20 cells, the ratio of survivin EX to FL also increased particularly at 24 and 48 hours. Although not significant the trends in the changes of splice variant ratio were consistent. There has been conflicting result as to the role of survivin in breast cancer, perhaps due to the expression of the different splice variants. For example one study looked at survivin with IHC and found that nuclear staining for survivin was an independent predictor of favourable prognosis (Kennedy *et al.*, 2003). Another study found that survivin was associated with a poor prognosis but nuclear and cytoplasmic localisation were not assessed separately (Hinnis *et al.*, 2007). In contrast another study found that survivin ΔEx3 was correlated positively with apoptosis (Ryan *et al.*,

2005). Due to the differing evidence it is plausible that FABP7 can have a detrimental or improved effect on prognosis depending on the role of survivin FL and ΔEx3 in breast cancer; further investigations are required to understand this. It is possible that FABP7 is shuttling various fats around the cell that lead to increased or decreased expression of the genes involved in apoptosis in the present study (Mita *et al.*, 2010; Liu *et al.*, 2012). The results for caspase 9 expression showed that there was little change in the ratio at any time point as a result of transfection, an indication that there is not a change in apoptotic properties. There were overall decreases in both caspase 9A and 9B mRNA though there was not a pattern as to whether they decreased as a result of the transfection or as result of FABP7 over-expression. Caspase 9 protein is an initiator caspase that is downstream of survivin; survivin interacts with caspase 9 through a phosphorylated domain to inhibit caspase 9 action/activation (Reed, 2001; Boatright & Salvesen, 2003). If survivin protein increased as mRNA did with FABP7 over-expression, this could result in a block on the apoptotic action of caspase 9. Alternatively FABP7 could inhibit apoptosis independently of caspase 9; for instance, considering the increased mitochondrial activity (measured by a MTT assay) in FABP7 over-expressing cells, FABP7 could alter mitochondrial activity and pathways involved in apoptosis perhaps by decreasing reactive oxygen species (ROS) that affect the mitochondrial membrane (Ott *et al.*, 2007; Fleury *et al.*, 2002). Without further investigation it is not possible to fully ascertain the relationship of FABP7 with apoptosis.

In the current study, evidence is presented to show that short term over-expression of FABP7 increases cell viability of breast cancer cells. Whether long term over-expression of FABP7 will confer the same effect remains to be investigated. Further studies using cell line models that enable long term investigation of FABP7 and patient cohorts with clinical follow up data will further aid understanding on whether FABP7 may be a novel prognostic marker in breast cancer.

5.6 Conclusions

FABP7 transfected BT-20 cells have increased viability compared to control vector transfected cells. FABP7 transfected cells are more resistant to death induced by FAs than control vector transfected cells. FABP7 transfection results in changes in survivin expression but further work is required to ascertain whether this is of prognostic value.

6 IGFBP-2 and PTEN expression in triple negative breast cancer

6.1 Introduction

IGFBP-2 is a member of the IGF-axis and modulates the action of insulin like growth factors (IGF-I, IGF-II) that principally signal via the type 1 IGF receptor (IGF-IR) (Perks *et al.*, 2007). The IGF axis has important roles in metabolism. IGFs, particularly IGF-II, have been found to be essential in foetal growth (Duan, 2002); links with the growth hormone axis and epidemiological studies have shown that IGFs are important in determining height and individuals who are taller are more at risk of developing cancer particularly in the breast or prostate (Okasha *et al.*, 2002). This axis plays a critical role in the development and progression of many epithelial cancers, including breast cancer (Meinbach & Lokeshwar, 2006). At the cellular level the IGF-I receptor appears to play a fundamental role in maintaining the transformed phenotype (Baserga *et al.*, 2003). Recent prospective epidemiology has consistently shown strong associations between circulating IGF-I levels and the subsequent risk of developing a number of epithelial cancers, including breast cancer (Roddam *et al.*, 2008). Whilst IGFBPs can act to either inhibit or enhance IGF-induced cell signalling they can also exert effects in an IGF-I-independent manner, indicating that IGFBPs can intrinsically modulate aspects of cell growth and survival (Holly & Perks, 2006). Busund *et al*. reported that in breast cancer, IGFBP-2 abundance was markedly higher in invasive breast carcinoma and carcinoma *in situ* when compared to normal breast tissue or benign hyperplastic lesions that had very little IGFBP-2 expression (Busund *et al.*, 2005). A study by Wang *et al.* showed that tumour expression of IGFBP-2 could predict tumours most likely to metastasise. IGFBP-2 clearly appears to play a role in breast cancer progression (Gebauer *et al.*, 1998; McGuire *et al.*, 1994; Wang *et al.*, 2000; Busund *et al.*, 2005; So *et al.*, 2008) and interestingly high levels of IGFBP-2 expression have also been identified in a number of additional cancers including those of the prostate (Degraff *et al.*, 2009), ovary (Yan *et al.*, 2009), stomach (Zhang *et al.*, 2007), adrenal

gland (Shi *et al.*, 2007) and bladder (Miyake *et al.*, 2005) suggesting that IGFBP-2 generally plays an important role in tumourigenesis. Although there has been much work investigating IGFBP-2 in general breast cancer cohorts and cell lines little if any work has investigated the relevance of IGFBP-2 in a cohort of TN breast cancer cases. However one study found that IGFBP-2 expression was associated with ER negativity in a general cohort (So *et al.*, 2008); further illustrating the importance of investigating IGFBP-2 in TN breast cancer. PTEN is a tumour suppressor gene that is lost or mutated in many types of cancer, including breast, prostate and lung cancer (Salmena *et al.*, 2008). PTEN dephosphorylates PIP_3, a product of the *PI3K* pathway, thereby inactivating the Akt signalling pathway, inhibiting cell growth and promoting apoptosis (Salmena *et al.*, 2008). More recently, PTEN has been postulated to have an important role in DNA repair, as mutation or loss of PTEN results in a deficiency to repair DNA double strand breaks (Shen *et al.*, 2007). Whilst one previous study has reported PTEN loss in 48% of unselected breast cancer cases, other studies have reported lower incidence of PTEN loss (8% (Panigrahi *et al.*, 2004), 15% (Perren *et al.*, 1999), 28% (Lopez-Knowles *et al.*, 2009)), though these differences may reflect methodological differences in testing and reporting PTEN loss. In contrast, a recent study suggests that up to 66% of basal like breast cancers have loss of PTEN and that PTEN loss may occur more frequently in this phenotype, compared to other subtypes of breast cancer (Lopez-Knowles *et al.*, 2009). An unbiased screen of human prostate and glioblastoma samples, using microarray-based expression profiling, identified IGFBP-2 as the most significant marker of PTEN loss (Mehrian-Shai *et al.*, 2007) and whilst the mechanism of loss of expression of PTEN in breast cancer has yet to be fully elucidated in cell lines PTEN activity is down-regulated by the interaction of IGFBP-2 with the *beta* 1 integrin receptor (Perks *et al.*, 2007) (section 1.7 and 1.8).

6.2 Aim and hypotheses

6.2.1 Aim

i) The purpose of the current study was to investigate for the first time the association between PTEN loss and IGFBP-2 expression in a cohort of TN breast cancer cases and relate this expression to basal cytokeratin expression and the clinicopathological features of stage, histological grade, patient age and overall survival.

6.2.2 Hypotheses

i) Loss of the PTEN gene is a frequent event in TN breast cancer and is associated with IGFBP-2 expression.

ii) IGFBP-2 expression and PTEN loss are associated with a worse prognosis in TN breast cancer.

6.3 Materials and Methods

Tissue blocks of all accessible triple negative breast carcinomas diagnosed between 2004 and 2009 at the University of Malaya Medical Centre, were used in this study, with a total of 101 identified as having adequate invasive tumour tissue for evaluation and were negative after re-assessment for ER, PR and Her2. All tissues had been fixed in 10% neutral buffered formalin between 6 to 72 hours, and processed to paraffin wax blocks, from which sections were cut at 3µM thickness on a rotary microtome and mounted onto Tissue Tek Plus glass slides to ensure maximum adhesion.

6.3.1 Assessment of Tumour Grade and Stage

Grading was according to the modified Bloom and Richardson criteria (Elston & Ellis, 1991) and all slides were reviewed and re-graded for this study by the histopathologists in the team. Clinical data on patient age, ethnicity and stage (American Joint Commission on Cancer, 2003), were extracted from the database for this series of cases. Patients were staged according to the 6th edition of the American Joint Commission on Cancer (AJCC) (Singletary & Connolly, 2006).

6.3.2 Immunohistochemistry

Expression of ER, PR, Her2, IGFBP-2, PTEN, cytokeratins 5/6 and cytokeratin 14 were tested for by IHC and staining assessed as described in section 2.1.

6.3.3 Statistical analysis

Continuous variables (age) were described using medians and compared using the Mann-Whitney U test, while categorical variables (ethnicity, stage, grade, IGFBP-2 expression, PTEN loss, CK5/6 expression and CK14 expression) were expressed as proportions and compared using either Chi

square test or Fisher's Exact test. Variables that were significantly associated with PTEN loss and IGFBP-2 expression were simultaneously entered into a multivariate logistic regression model with PTEN loss and IGFBP-2 as the outcome variables, to determine the independent predictors of PTEN loss and IGFBP-2 expression. Considering that information on cause of death was not available for the majority of patients, the relative survival rates (RSRs) were calculated to estimate the excess mortality among the patient population due to breast cancer. Population mortality data for Malaysia was used to compute these estimates. The relative survival adjusts for the general survival of the Malaysian population for the given sex, age and year, and thus is a measure of net survival attributed to breast cancer independent of other causes of death. P values of less than 0.05 and 95% confidence interval (CI) for odds ratio (OR) which does not include 1.0 were considered as statistically significant. All statistical analyses were carried out using SPSS IBM Statistics (Version 20) and Stata MP (Version 14). Analysis for the study was carried out by Dr. Nirmala Boo Pathy.

6.4 Results

Following re-testing of ER, PR and Her2 and confirmation of the triple negative status of the breast cancers, a total of 89 cases were available for analysis of PTEN loss and basal cytokeratin expression and a total of 100 available for analysis of IGFBP-2 and basal cytokeratin expression. The age of onset of breast cancer in the patients ranged from 23 to 83 years old with a median of 53 years. The majority of cases (93%) were invasive ductal carcinomas, with the remaining being either medullary or metaplastic cancers.

6.4.1 IGFBP-2

IGFBP-2 staining was predominantly confined to the cytoplasmic compartment of the invasive cancer cells with little stromal staining or expression in normal glands. Heterogeneity of staining, with some invasive tumour cells showing strong expression for IGFBP-2 and other cells showing little or no expression was a common feature. Addition of the IGFBP-2 blocking peptide resulted in complete absence of staining in cases shown to be strongly positive for IGFBP2 (figure 6.4.1).

One hundred TN cases were assessable for IGFBP-2 staining with 32% showing high levels of IGFBP-2 expression (Allred score >5). Univariate analysis revealed no significant differences in high IGFBP-2 expression and age, stage, or CK5/6 but there was an association between IGFBP-2 expression and lack of staining for CK14 (p=0.004) (table 6.1). In the multivariate analysis, independent predictors of high levels of IGFBP-2 expression were lack of positivity for CK14 (p=0.005) (figure 6.2) and PTEN loss (p=0.047) (figure 6.3). There was also a trend for lympho-vascular invasive cases to have high levels of IGFBP-2 expression but this trend was not significant (p=0.064) (table 6.2).

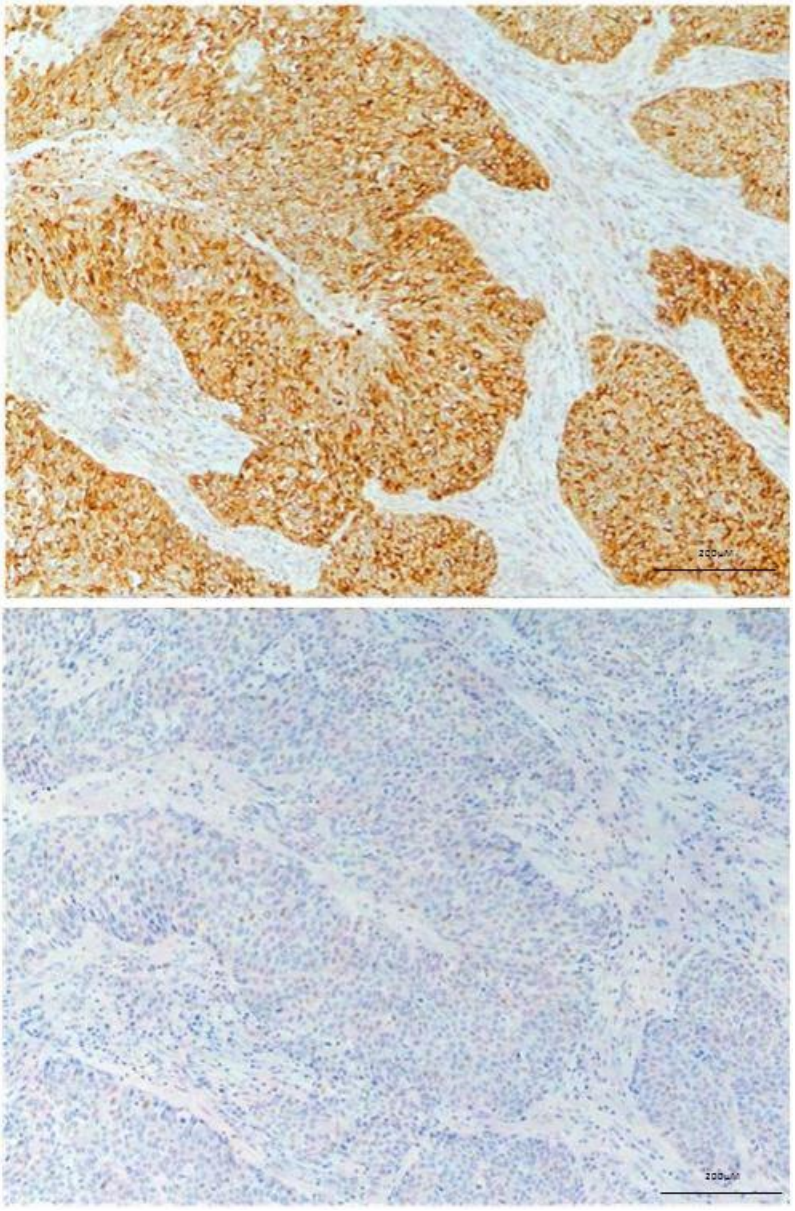


Figure 6.1 IGFBP-2 staining pattern and the specificity of the IGFPB-2 antibody

Top: Immunohistochemical cytoplasmic staining for insulin like growth factor binding protein-2 (IGFBP-2) in a triple negative invasive ductal carcinoma. Bottom: The addition of the IGFBP-2 blocking peptide resulted in complete absence of cytoplasmic staining for IGFBP-2 of this tumour.

Table 6.1 Factors associated with IGFBP-2 expression

	Overall N (%)	*IGFBP2 positive N (%)*	*IGFBP2 negative N (%)*	*P for X^2 test*
No. of patients	100	32	68	
Age (median, years)	53	52	53	0.685[a]
Ethnicity				0.215
Chinese	63 (63.0)	19 (59.4)	44 (64.7)	
Malay	21 (21.0)	6 (18.8)	15 (22.1)	
Indian	13 (13.0)	7 (21.9)	6 (8.8)	
Others	3 (3.0)	0 (0.0)	3 (4.4)	
Tumour size (median, cm)	3.0	3.3	3.0	0.445[a]
Lymph node involved				0.866
Yes	44 (44)	15 (42.9)	29 (44.6)	
No	56 (56)	20 (57.1)	36 (55.4)	
Stage				0.837[b]
Early	65 (65)	24 (68.6)	41 (63.0)	
Late	35 (35)	11(31.4)	24 (37.0)	
Grade[c]				0.855
Grade 2	19 (19.6)	7 (20.6)	12 (19)	
Grade 3	78 (80.4)	27(79.4)	51 (81)	
Unknown	3	1	2	
Lymphovascular invasion				0.176
Present	34 (34.3)	16 (47.1)	18 (32.7)	
Absent	55 (65.7)	18 (52.9)	37 (67.3)	
Unknown	11	1	10	
CK14 status				0.004
Negative	55 (56.7)	26 (76.5)	29 (46)	
Positive	42 (43.3)	8 (23.5)	34 (54)	
Unknown	3	1	2	
CK 5/6 status				0.368
Positive	26 (29.5)	11 (35.5)	15 (26.3)	
Negative	62 (70.5)	20 (64.5)	42 (73.7)	
Unknown	**12**	4	8	
PTEN loss				0.073
Yes	43 (48.3)	19 (61.3)	24 (41.4)	
No	46 (51.7)	12 (38.7)	34(58.6)	
Unknown	11	1	10	

Key:

a) Compared using Mann-Whitney-U test
b) Compared using Fisher's Exact test
c) No patients with grade 1 tumour
d) Defined as an Allred Score >5.

Table 6.2 Factors associated with IGFBP-2 expression in multivariate analysis

Factors	*Odds ratio for IGFBP2 positivity* [a] *(95% confidence interval)*	*95% confidence interval for odds ratio*		*Significance (p)*
		Lower	*Upper*	
Lympho-vascular invasion				
Absent	1.00 [b]			
Present	2.56 [c]	0.95	6.93	0.064
CK 14 expression				
No	1.00 [b]			
Yes	0.23 [c]	0.08	0.64	0.005
PTEN loss				
No	1.00			
Yes	2.74 [c]	1.02	7.39	0.047

KEY: (a) derived using a multivariable logistic regression model including all variables with p value < 0.20 in univariable analysis, lymphovascular invasion, CK 14 expression, and PTEN loss, (b) reference category, (c) statistically significant (p< 0.05)

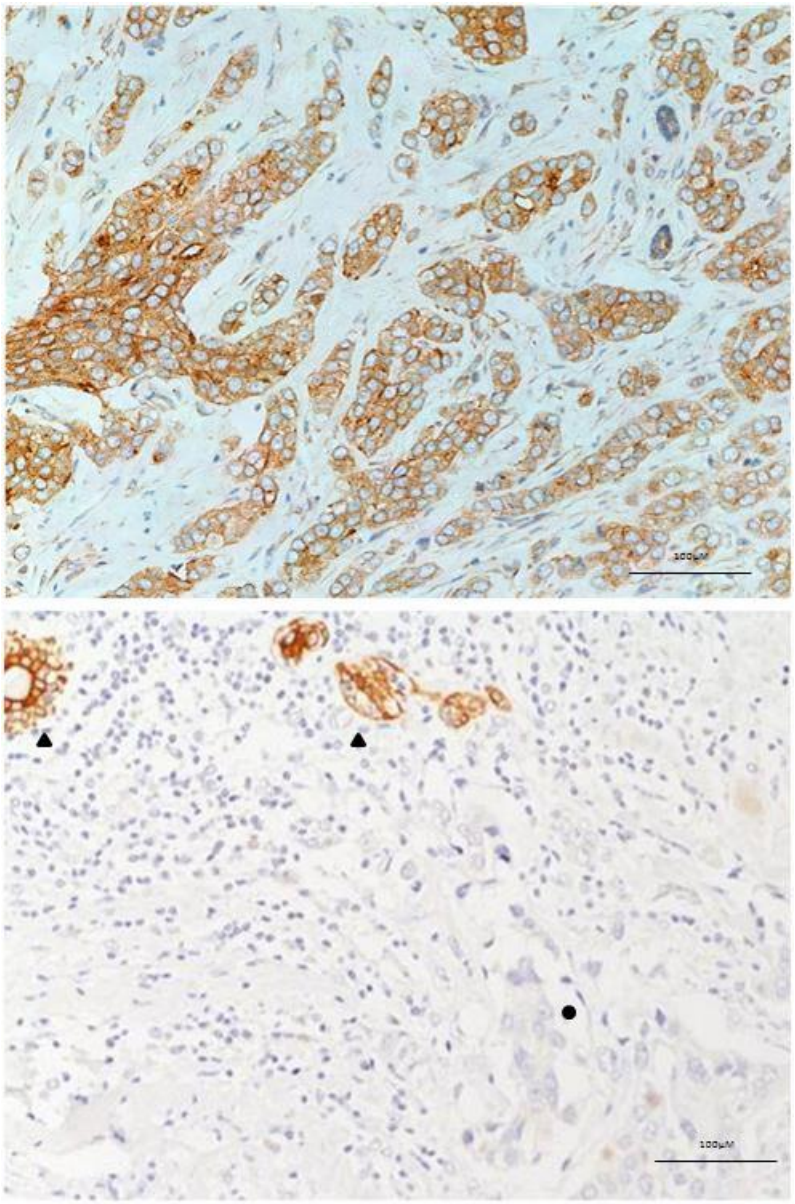


Figure 6.2 The association of IGFBP-2 expression and absence of CK14 expression

Top: x20 A triple negative infiltrating ductal carcinoma immunohistochemically stained for insulin like growth factor binding protein-2 (IGFBP-2). Bottom: x40 The same tumour immunohistochemically stained for Cytokeratin 14 whilst the normal glands stain positively ◄, the invasive tumour component is negative ●.

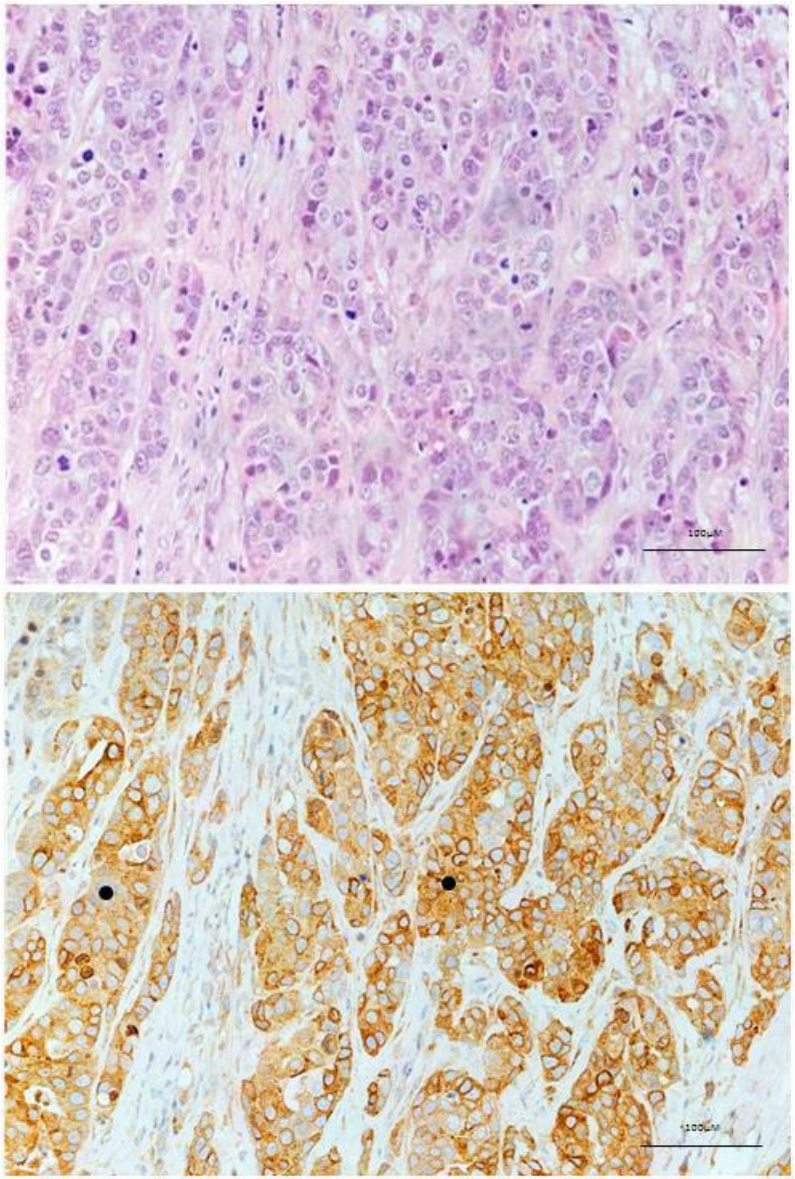
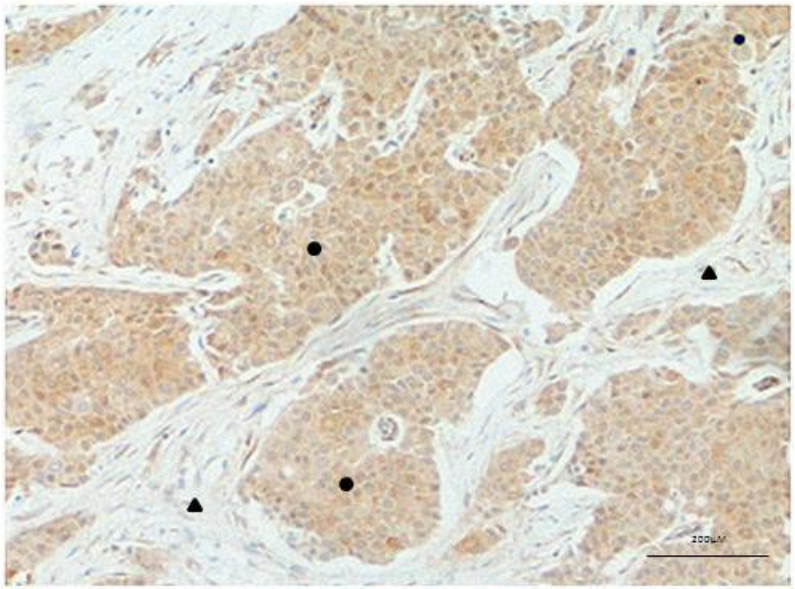


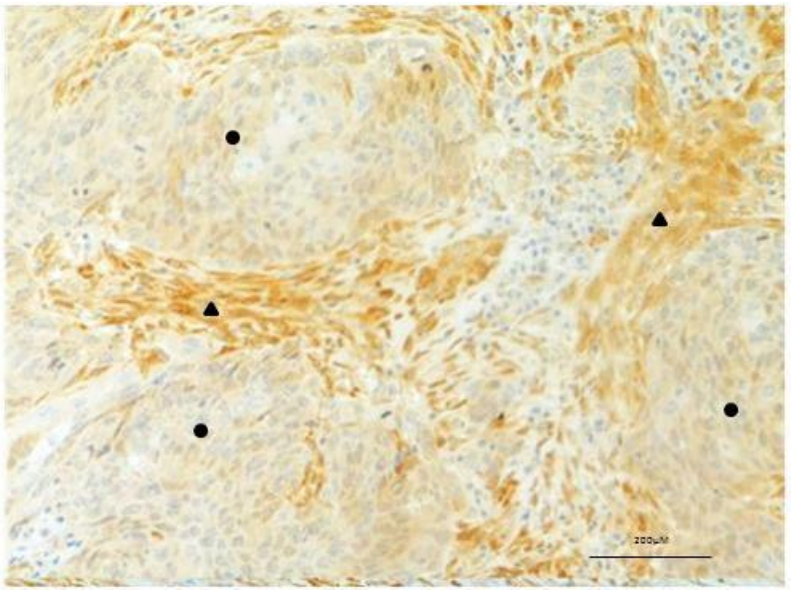
Figure 6.3 The association of IGFBP-2 expression and PTEN loss

Top Left: x20 A triple negative IDC stained with Haematoxylin and Eosin Top Right: x20 the same IDC showing loss of PTEN staining in tumour cells ● and positive surrounding stromal tissue ◄. Bottom: x20 Strong cytoplasmic staining for IGFBP-2 in tumour cells ● of the same case.

6.4.2 PTEN

PTEN staining occurred in both the nucleus and cytoplasm of cancer cells. Loss of nuclear and cytoplasmic immunostaining for PTEN occurred in 48.3% of triple negative cases; the remaining cases had weak PTEN staining or stronger staining of PTEN equal to that of the surrounding stromal tissue (figure 6.4). Univariable analysis showed that loss of PTEN immunostaining was associated with younger age of onset; the median age of patients at diagnosis with tumours showing PTEN loss was 47 years old, compared with 57 years in those without PTEN loss (p=0.005). PTEN loss was also associated with CK5/6 expression and IGFBP-2 expression, though these trends were not statistically significant in univariable analysis (p=0.097 and p=0.073 respectively) (table 6.3). In multivariable analysis, independent predictors of PTEN loss were younger age of onset (p=0.041), late stage (p=0.026), CK5/6 positivity (p=0.028) and IGFBP2 expression (p=0.042) (table 6.4) (figures 6.3 & 6.5).





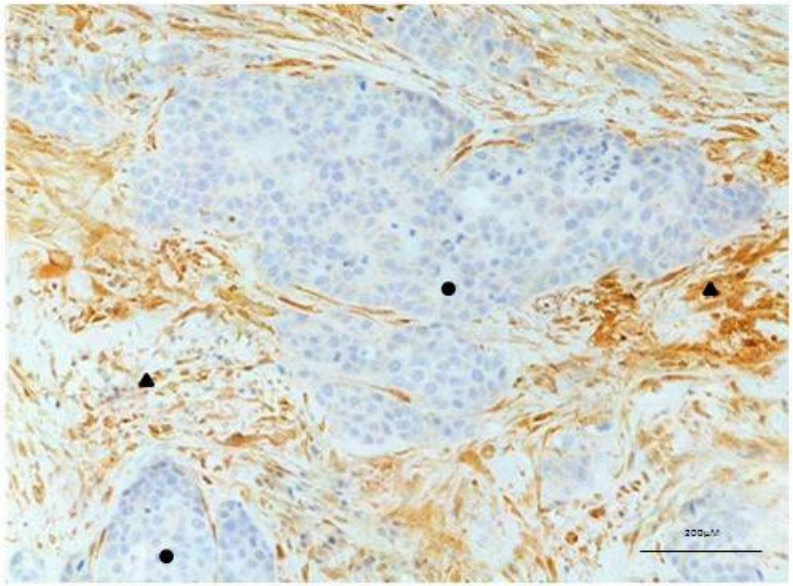


Figure 6.4 The types of PTEN staining

Patterns of immunohistochemical staining for PTEN in three triple negative invasive breast carcinomas (IDC). Top left: x10 Cytoplasmic and nuclear staining of PTEN in the IDC tumour cells ● and positive surrounding stromal tissue ◄. Top right: x10 Weak cytoplasmic and nuclear staining for PTEN in the IDC cells ● compared to strong staining in the surrounding stromal tissue ◄. Bottom: x10 Absence of staining for PTEN in the IDC, indicating total loss of PTEN in the tumour compartment ●, with strong staining for PTEN in the adjacent stromal tissue ◄.

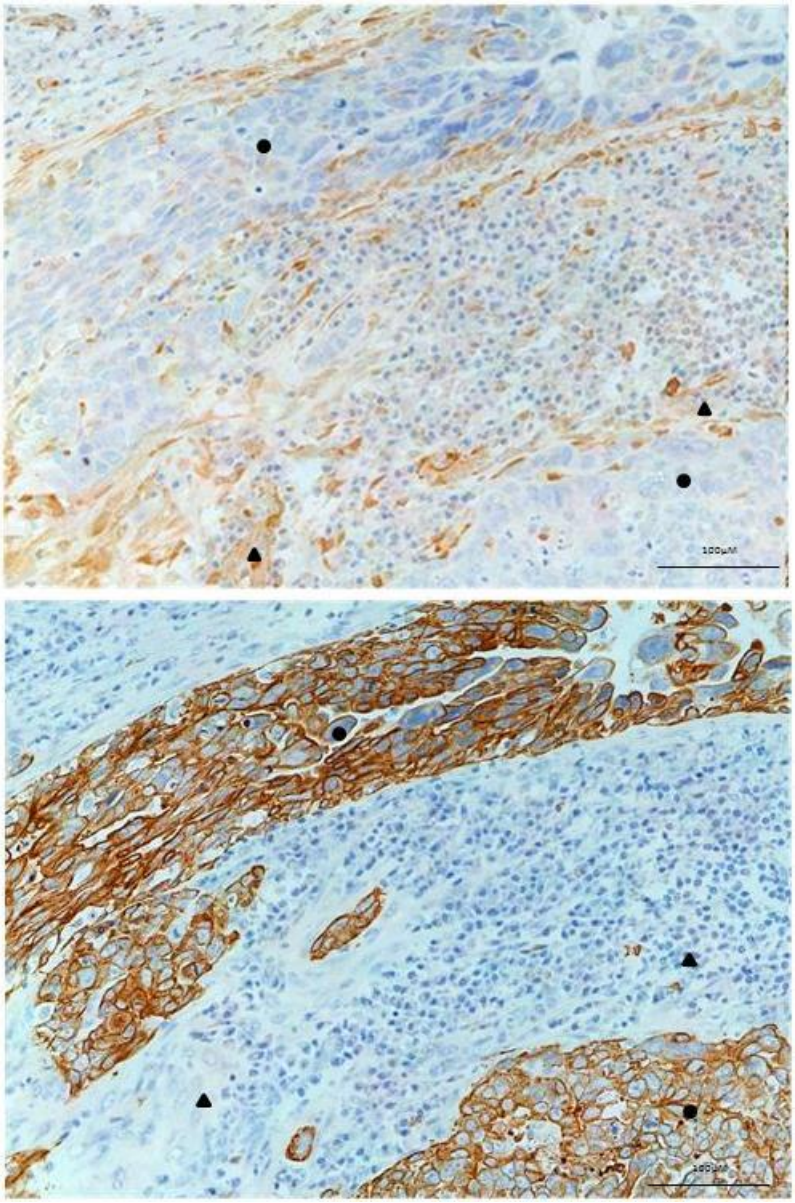


Figure 6.5 The association of PTEN loss with CK5/6 expression

Top: x20 Loss of immunohistochemical staining for PTEN ● in a triple negative IDC with PTEN positivity in surrounding stromal tissue ◄. Bottom: x20 Strong cytoplasmic staining for cytokeratin 5/6 ● in the same tumour with negative staining in surrounding stromal tissue◄.

Table 6.3 Factors associated with PTEN expression

	Overall N (%)	*PTEN loss N(%)*	*No PTEN loss N(%)*	*P for X^2 test*
No. of patients	89	43	46	
Age (median, years)	53	47	57	0.005 [a b]
Ethnicity,				0.518 [c]
Chinese	53 (59.6)	27 (62.8)	26 (56.5)	
Malay	21 (23.6)	10 (23.3)	11 (23.9)	
Indian	12 (13.5)	6 (14.0)	6 (13.0)	
Others	3 (3.4)	0 (0.0)	3 (6.5)	
Tumour size (median, cm)	3.0	3.5	3.0	0.236 [b]
Lymph node involved,				0.250
Yes	42 (47.2)	23 (53.5)	19 (41.3)	
No	47 (52.8)	20 (46.5)	27 (58.7)	
Stage,				0.078
Early (Stage 1-2)	55(61.8)	23(53.5)	32(69.6)	
Late (Stage 3-4)	34(38.2)	20(46.5)	14(30.4)	
Grade [d],				0.145
Grade 2	16 (18.6)	5 (12.2)	11(24.4)	
Grade 3	70 (81.4)	36 (87.8)	34 (75.6)	
Unknown	3	2	1	
Lymphovascular invasion,				0.780
Present	32 (40.5)	16 (42.1)	16 (39)	
Absent	47 (59.5)	22 (57.9)	25 (61)	
Unknown	10	5	5	
CK14 status,				0.354
Negative	47 (54)	20 (48.8)	27 (58.1)	
Positive	40 (46)	21 (51.2)	19 (41.3)	
Unknown	87	2	0	
CK 5/6 status,				0.097
Negative	23 (29.1)	8 (20.5)	15 (37.5)	
Positive	56 (70.9)	31 (79.5)	25 (62.5)	
Unknown	10	4	6	
IGBFP2,				0.073
Positive [e]	31 (34.8)	19 (44.2)	12 (26.1)	
Negative	58 (65.2)	24 (55.8)	34 (73.9)	

Key:

a) Statistically significant (p less than 0.05)
b) Compared using Mann-Whitney-U test
c) Compared using Fisher's Exact test
d) There were no patients with grade 1 tumour
e) Defined as an Allred Score of >5.

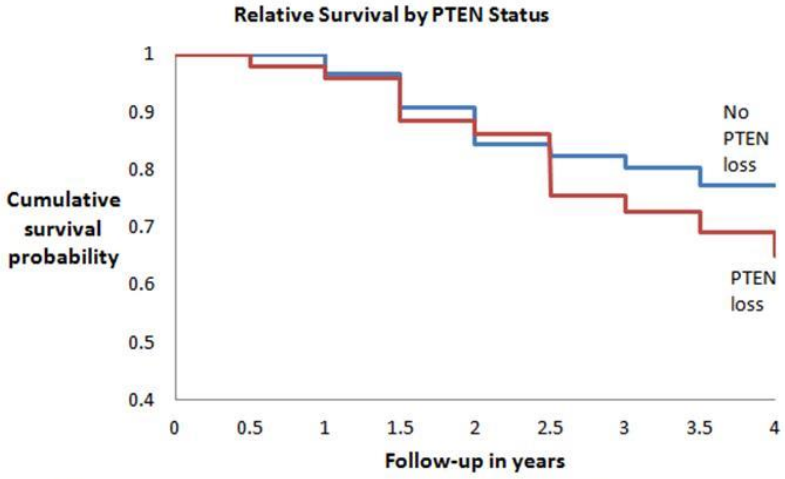
Table 6.4 Factors associated with PTEN expression in multivariate analysis

Factors	*Odds ratio for PTEN loss[a] (95% confidence interval)*	*95% confidence interval for odds ratio*		*Significance (p)*
		Lower	*Upper*	
Age (years)	0.95 [b]	0.91	1.00	0.041
Stage				
Early (Stage 1-2)	1.00 [c]			
Late (Stage 3-4)	3.76 [b]	1.17	12.10	0.026
Grade				
Grade 2	1.00 [c]			
Grade 3	2.95	0.81	10.77	0.101
CK 5/6 expression				
No	1.00 [c]			
Yes	3.94 [b]	1.16	13.34	0.028
IGBFP2 expression				
No	1.00 [c]			
Yes	3.26 [b]	1.04	10.21	0.042

KEY: (a) Derived using a multivariable logistic regression model including all variables with p value < 0.20 in univariable analysis; age, stage, grade, CK 5/6, and IGBFP2 status, (b) Statistically significant ($p<0.05$), (c) Reference category

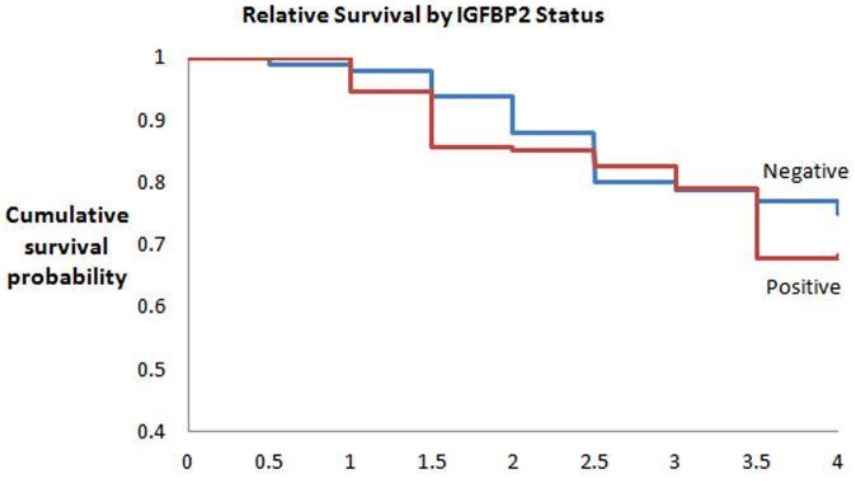
6.4.3 Survival analysis

Triple negative cases with PTEN loss tended to have a worse survival compared to cases without PTEN loss; 4-year relative survival rate (RSR) for patients with PTEN loss was 65.0% (95%CI: 45.2-79.7%) as compared to 77.8% (95%CI: 60.8-89.0%) in those without PTEN loss; though this was not significant (figure 6.6). Similarly, cases with high levels of IGFBP-2 experienced marginally lower survival than their counterparts with low or negative IGFBP-2; 4-year RSR; 68.4% (95%CI: 44.5-84.5) compared to 74.9% (95%CI:60.8-85.1) (figure 6.7), however this was not significant.



PTEN Status	Year(s) following diagnosis with breast cancer				
	0	1	2	3	4
No PTEN Loss					
No of patients entering interval	46	44	38	26	18
Relative survival,% (95%CI)	100	96.7 (84.5-0.99)	84.3 (69.5-92.8)	80.3 (64.6-90.3)	77.8 (60.8-89.0)
PTEN loss					
No of patients entering interval	43	37	33	20	13
Relative survival,% (95%CI)	100	95.7 (81.9-99.5)	86.2 (70.4-94.5)	72.7 (54.7-84.9)	65.0 (45.2-79.7)

Figure 6.6 PTEN expression and patient samples
Kaplan Meier survival analysis was carried out for PTEN negative versus PTEN positive cases. Results show that PTEN positive cases had better survival than cases that were PTEN negative.



IGFBP2	Year(s) following diagnosis with breast cancer				
	0	1	2	3	4
Negative					
No of patients entering interval	68	64	60	47	34
Relative survival,% (95%CI)	100	97.9 (89.2-100.0)	87.8 (76.5-94.4)	78.7 (65.8-87.7)	74.9 (60.8-85.1)
Positive					
No of patients entering interval	32	31	26	24	12
Relative survival,% (95%CI)	100	94.5 (77.3-99.4)	85.6 (67.0-94.8)	79.0 (58.8-90.8)	68.4 (44.6-84.5)

Figure 6.7 IGFBP-2 expression and patient survival

Kaplan Meier survival analysis was carried out for IGFBP-2 negative versus IGFBP-2 positive cases. Results show that IGFBP-2 positive cases had poorer survival than cases that were IGFBP-2 negative.

6.5 Discussion

6.5.1 IGFBP-2 expression in TN breast cancer

The IGFBP-2 staining pattern in the present study was similar to that noted by Busund *et al*. (2005) who also looked at IGFBP-2 expression in breast cancer. An Allred score of 5 was used as a cut off point for IGFBP-2 positivity; using this cut off point 32% of cases were positive for IGFBP-2. This is a similar percentage to another study using an unselected breast cancer cohort; 37% of cases were moderate to strongly positive for IGFBP-2 So *et al.* (2008). As the percentages are similar in both TN and unselected breast cancer cohorts, IGFBP-2 positivity may not be related to hormone receptor or Her2 status. IGFBP-2 positivity was associated with lymphovascular invasion and worse breast cancer survival though neither of these trends reached statistical significance; possibly due to the small number of cases in the current study. This data is consistent with that of So *et al*., who showed that of 3117 breast tumours that were assessable for both ERα status and IGFBP-2 expression, IGFBP-2 was not prognostic among the ERα positive tumours, but a trend was revealed towards worse breast cancer disease-specific survival in the ER negative tumours. However the study did not distinguish between Her2 positive and TN cases within in the ERα negative group. Busund *et al*., (2005) also found that IGFBP-2 expression was higher in benign hyperplasia compared to normal tissue and that IGFBP-2 expression was highest in malignant breast cancer. Furthermore, *in vitro* studies have demonstrated that over-expression of IGFBP-2 in ER negative breast cancer cell lines and cell lines of other cancer types to include those of prostate, glioma and bladder, conferred a growth advantage, enhanced invasion and migration and chemoresistance (Kiyama *et al.*, 2003; Miyake *et al.*, 2005; So *et al.*, 2008; Wang *et al.*, 2008). Interestingly IGFBP-2 expression in tumours was linked to lack of staining for CK14. TN breast cancers are enriched for characteristics of epithelial mesenchymal transition (EMT) or mesenchymal epithelial transition (MET); changes in CK14 expression can be indicative of EMT or MET (Hugo *et al.*, 2007; Mani *et al.*, 2008; Savagner, 2010). It can be speculated that these tumours may be undergoing EMT or MET and that IGFBP-2 may be involved. However, further

studies would be required to confirm this. Perhaps using additional cytokeratin markers would help further understand the relationship between IGFBP-2 and basal like phenotype. A recent study showed that IGFBP-2 promotes angiogenesis in neuroblastoma cells via direct activation of the VEGF promoter (Azar *et al.*, 2011) and anti-VEGF therapy of gliomas, infiltrating tumour was associated with increased levels of IGFBP-2 (de Groot *et al.*, 2010). VEGF can be targeted and clinical trials have taken place using such drugs against VEGF in TN breast cancer. For example bevacizumab a monoclonal antibody against VEGF has shown promise when used with cisplatin against TN breast cancer (Ryan *et al*., 2009); perhaps co-targeting IGFBP-2 and VEGF might be of benefit in TN breast cancer if IGFBP-2 is further demonstrated to be associated with poor prognosis. In addition over-expression of EGFR and IGFBP-2 has been observed in high grade astrocytomas and co-expression of these genes was strongly associated with high grade gliomas and lower survival. This report suggested that co-expression of these genes had a more important clinical and biological impact than the expression of each individual gene alone (Scrideli *et al.*, 2007). EGFR is strongly associated with TN breast cancer and is a potential target for therapy (Sarrio *et al.*, 2008; Gluz *et al.*, 2009); there may now be rationale for assessing EGFR status in relation to IGFBP-2 expression in TN breast cancers as perhaps co-targeting both of IGFBP-2 and EGFR for example with, monoclonal antibody therapy, would provide a better outcome.

6.5.2 PTEN loss of expression in TN breast cancer

Loss of PTEN staining in the invasive tumour compartment was readily assessable, as strong positive staining of the adjacent non-tumour stroma serves as an excellent internal positive control, as previously reported (Perren *et al.*, 1999). In the current study, PTEN expression in tumour cells is lost in nearly half of TN breast cancers using the antibody clone 6H2.1. Notably, the extent of PTEN loss in triple negative breast cancer was significantly higher than that reported utilising the same PTEN antibody in an unselected breast cancer cohort (Lopez-Knowles *et al.*,

2009). Clone 6H2.1 is the recommended marker for immunohistochemical analysis of PTEN loss as it is the only antibody that exhibits a correlation with molecular alterations in PTEN (Pallares *et al.*, 2005) and shows correlation between western blot analysis and PTEN mutational and allelic status (Perren *et al.*, 1999). Another reason for the extent of PTEN loss could be due to differences in the type of cohort. The cohort in the present study was of Asian origin; breast cancers in Malaysia tend to be more aggressive than in other countries (section 1.9). Dietary and lifestyle factors have been suggested as reasons for these differences. However with respect to PTEN loss a study looking at PTEN loss and mutations in colorectal cancer found no correlation with dietary factors (Naguib *et al.*, 2011). One way of investigating whether the frequency of PTEN loss is high in TN breast cancer or associated with Malaysian breast cancer would be to use IHC staining for PTEN on a local UK TN breast cancer cohort. Interestingly, loss of PTEN was significantly associated with a younger age at diagnosis. This reflects the findings by Anders *et al.* (2008) where PTEN expression and genes involved in related signalling pathways were altered in breast cancers that occurred in patients 45 years old and younger. Collaborative work has led to publication of the association of PTEN loss with age and has also demonstrated that PTEN status assessed by IHC increases the sensitivity of the Manchester score analysis. The Manchester score analysis uses criteria including grade, hormone and Her2 receptor status to predict *BRCA1* and *BRCA2* mutations (Phuah *et al.*, 2012).

There was a significant association of PTEN loss with CK5/6 positivity. This probably reflects the association of PTEN loss with basal-like phenotype and is in conjunction with other studies (Marty *et al.*, 2008; Neto *et al.*, 2012). Although not significant, there was an association between PTEN loss and reduced breast cancer survival; this is in agreement with significant findings by Depowski *et al* (2001) in a cohort of breast cancers not selected on the basis of TN status. TN cohorts generally tend to have poorer survival than unselected cohorts of breast cancer cases. Consequently, it is probably necessary to study larger TN cohorts with PTEN loss in order to

establish whether the trend observed in the current study becomes significant, when larger numbers of cases are included.

6.5.3 IGFBP-2 expression is associated with PTEN loss

This is the first immunohistochemical study to show an association between IGFBP-2 expression and PTEN loss and supports the evidence from *in vitro* cell line studies that show IGFBP2 down-regulates PTEN (Perks *et al.*, 2007) and conversely that over-expression of PTEN has been shown to reduce IGFBP-2 expression (Levitt *et al.*, 2005). These studies indicate IGFBP-2 plays a role in the *PI3K* signalling pathway that is known to be involved in promoting survival and growth (Hers *et al.*, 2011). A recent study has investigated the relationship between PTEN loss and IGFR-1 expression in TN breast cancer cases; it was found that PTEN loss was associated with IGFR-1 over-expression and that together these markers may be useful in predicting reoccurrence in TN breast cancer and may prove useful targets of therapy. This provides further evidence of the overlap between the IGF axis, PTEN and the *PI3K* pathways (Iqbal *et al.*, 2012). There are several potential pathways that could be behind this inverse relationship; one is that increased IGFBP-2 expression by breast cancer cells could partially diminish or ablate the expression of PTEN protein, potentially via interaction with integrin receptors (Perks *et al.*, 2007). Further work could also investigate how IGFBP-2 impacts on the catalytic activity of PTEN. PTEN possesses a carboxy-terminal, non-catalytic regulatory domain with three phosphorylation sites (Ser380, Thr382 and Thr383) that regulate its biological activity (Vazquez *et al.*, 2000; Torres *et al.*, 2001). Antibodies are available that recognize phosphorylation at these sites and may prove useful in further studies to investigate the relationship between IGFBP-2 expression and PTEN phosphorylation and thus activity.

6.6 Conclusions

In summary this chapter has shown that IGFBP-2 over expression is a frequent event in TN breast cancer and was associated with poor prognosis, though this trend was not significant. It has shown that loss of PTEN can be readily assessed by immunohistochemistry and that PTEN loss is a frequent event in triple negative breast cancers. PTEN loss is significantly associated with a younger age of onset of breast cancer and late stage of presentation. This is the first immunohistochemical study to show an association between IGFBP-2 expression and PTEN loss and supports the evidence from *in vitro* cell line studies that show IGFBP2 down-regulates PTEN.

7 General Discussion

Breast cancer is the most common type of cancer in the UK and current guidelines recommend that all cases be tested for expression of the biomarkers of ER, PR and Her2 (Wolff *et al.*, 2007; Hammond *et al.*, 2010). These subtypes of breast cancer can be treated with targeted therapies such as tamoxifen and trastuzumab for ER and Her2 positive breast cancer, respectively. However, some breast cancers do not express ER, PR or Her2 and are consequently classified as triple negative breast cancer and targeted therapy is not available for this type of disease. To define any cancers by the markers they do not express will never be completely satisfactory. One aim of this research was to investigate new biomarkers of relevance in TN breast cancer and relate biomarker expression to patient and clinicopathological features. A further aim was to investigate the expression of a particular biomarker of interest, FABP7, in cell line models and relate expression of FABP7 to features such as survival and apoptosis in cell lines.

Expression profiling and IHC studies have identified FABP7 to be over-expressed in basal-like breast cancer, and consequently TN breast cancer, as over 80% of basal like breast cancer have been found to lack expression of ER, PR and Her2. However the significance of FABP7 with prognosis in breast cancer is unclear and studies have given conflicting evidence (Zhang *et al.*, 2010; Tang *et al.*, 2010). The *in vivo* patient sample work (chapter 3) sought to investigate the association of FABP7 with clinicopathological features in a cohort of Malaysian TN breast cancer cases. It was found that FABP7 expression was associated with lower breast cancer stage and the basal-like phenotype. There was also a trend for cases with high levels of FABP7 to have improved survival, though this trend was not significant, they are in agreement with an earlier study in an unselected cohort (Zhang *et al.*, 2010). The results also suggest that the role of FABP7

expression is similar in both Malaysian and western breast cancer cohorts. One way of confirming any similarities is to compare FABP7 staining frequency in the Malaysian cohort to a UK breast cancer cohort, using the same FABP7 antibody; work has been undertaken to begin this investigation. A longer follow-up period and perhaps larger cohorts, comparing the UK and Malaysian cohorts, may help confirm the relationship between FABP7 expression and breast cancer survival.

In the current study, interestingly FABP7 and FAS expression were positively associated suggesting a link between fatty acid metabolism and FABP7 expression; further work would involve investigating the relationship in a UK TN breast cancer cohort and to use cell line models to investigate whether the expression of FAS is influenced by FABP7 expression or *vice versa*. The aim of the study was also to investigate FABP7 mRNA and protein in breast cancer cell lines in order to choose an appropriate model for further investigation of FABP7. BT-20 cells were found to express the highest amount of FABP7 mRNA and protein out of the breast cancer cell lines investigated; BT-20 cells are of basal like phenotype so this was anticipated. However, mRNA expression was lower overall than expected and FABP7 protein was not detectable without a highly modified western blot protocol. Whilst expression was highest in the BT-20 cell line, it was not expressed at high enough levels suitable for knock-down experiments. Given the high level of FABP7 expression amongst the TN cases with the basal like phenotype, it was expected that at least one of the cell lines expressing a similar phenotype (BT-20, MDA-MB-231, MCF10A, and HS578T) may have levels of FABP7 at sufficient level to allow knock-down. This may explain the paucity of *in vitro* cell line studies utilising a breast cancer cell line that naturally expresses FABP7. Other studies have had similar findings; one study used the MDA-MB-435 cell line to knock-down FABP7 (Liu *et al.*, 2012) though evidence suggests that this cell line may be of melanoma origin rather than breast cancer origin (Rae *et al.*, 2007; Lacroix, 2009). A limiting factor may be the

number of cell lines tested; each cell line would have been originally derived from one patient; nine cell lines may not be a large enough selection to be representative of FABP7 expression in clinical cases. Though notably based on IHC results it was expected that at least one of the basal-like cell lines investigated would express FABP7. Alternatively, there might be factors absent *in vitro* that are essential for FABP7 expression; for example, fat exposure or the epigenetic status of FABP7 may be different in the cell lines used in the present study compared to patient samples.

Another aim was to more fully understand the mechanisms that regulate FABP7 expression and thus identify factors that may or may not be occurring *in vitro*; this was investigated by treating BT-20 and MDA-MB-231 cells with AZA, TSA and the FAs oleate, palmitate, AA and DHA (chapter 4). There is an abundance of evidence suggesting that FABP7 is regulated by epigenetic mechanisms (reviewed in section 1.6.1) but little if any work has looked at whether methylation or acetylation are factors associated with FABP7 expression in breast cancer cell lines. AZA significantly increased FABP7 mRNA expression but not to a level that was expected. If FABP7 was methylated directly then larger fold changes may have been expected, perhaps similar to fold changes of ER re-expression with AZA treatment in breast cancer cell lines (Yang *et al.*, 2001). It was therefore concluded that FABP7 was indirectly regulated by methylation and acetylation. A combined bisulfite restriction analysis (COBRA) assay may have been useful in confirming whether FABP7 was methylated but CpG islands could not be identified in the FABP7 promoter region. However methylation can occur at other regions of the gene and at regions other than CpG islands (Wozniak *et al.*, 2007). Possible mechanisms for the increased expression of FABP7 with AZA treatment are outlined in figure 7.1; a transcription factor of FABP7, for instance STAT3 could be methylated or a regulator of the transcription factor such as cAMP response element binding protein (CREB) could be methylated thus preventing FABP7 expression in both instances.

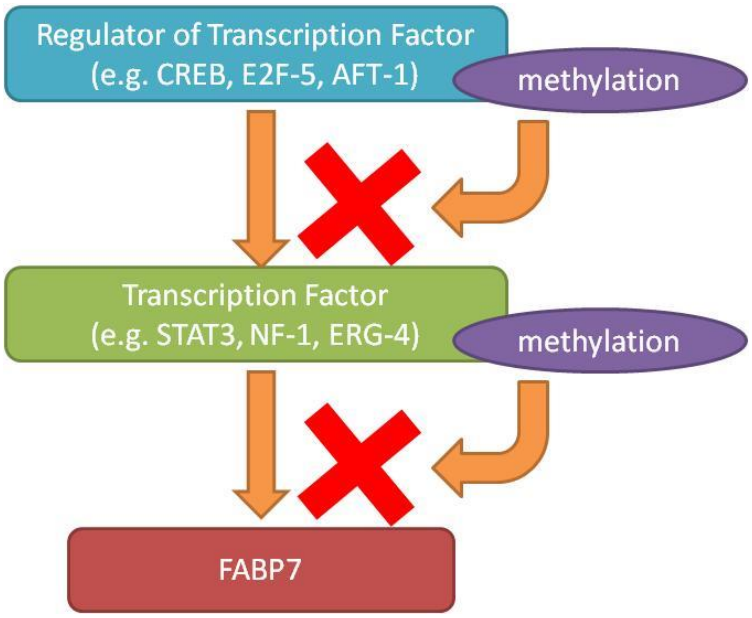


Figure 7.1 The indirect mechanisms by which methylation could alter FABP7 expression

A transcription factor, such as STAT3, NF-1 or ERG-4, upstream of FABP7 is re-expressed after AZA treatment resulting in an increase in FABP7 expression. Alternatively a regulator of a transcription factor such as cAMP response element binding protein (CREB) for STAT3, E2 factor-5 (E2F-5) for NF-1 and activating transcription factor-1 (AFT-1) for ERG-4, is demethylated after AZA treatment leading to increased expression of both the transcription factor (e.g. STAT3) and in turn FABP7.

The results of chapter 3 also showed that FA treatments on their own resulted in small but significant changes of FABP7 mRNA expression. However when FAs were combined with AZA treatment significant increases in FABP7 expression resulted. Firstly, the double dose of AZA significantly increased FABP7 mRNA expression and it was postulated that this was due to the indirect nature of methylation impacting on FABP7 expression; longer time of exposure and a higher dose of AZA is required to activate and re-express targets upstream of FABP7. When AZA was combined with FA treatment palmitate synergistically increased FABP7 expression and oleate, AA and DHA decreased FABP7 mRNA compared to AZA treatment alone. The reasons that have been put forward in light of these results are that palmitate may facilitate the binding of transcription factors or co-activator to genes that are re-expressed after AZA treatment. Oleate, AA and DHA prevent binding of co-activators and transcription factors to genes after demethylation by AZA treatment. This hypothesis is summarised in figure 7.2

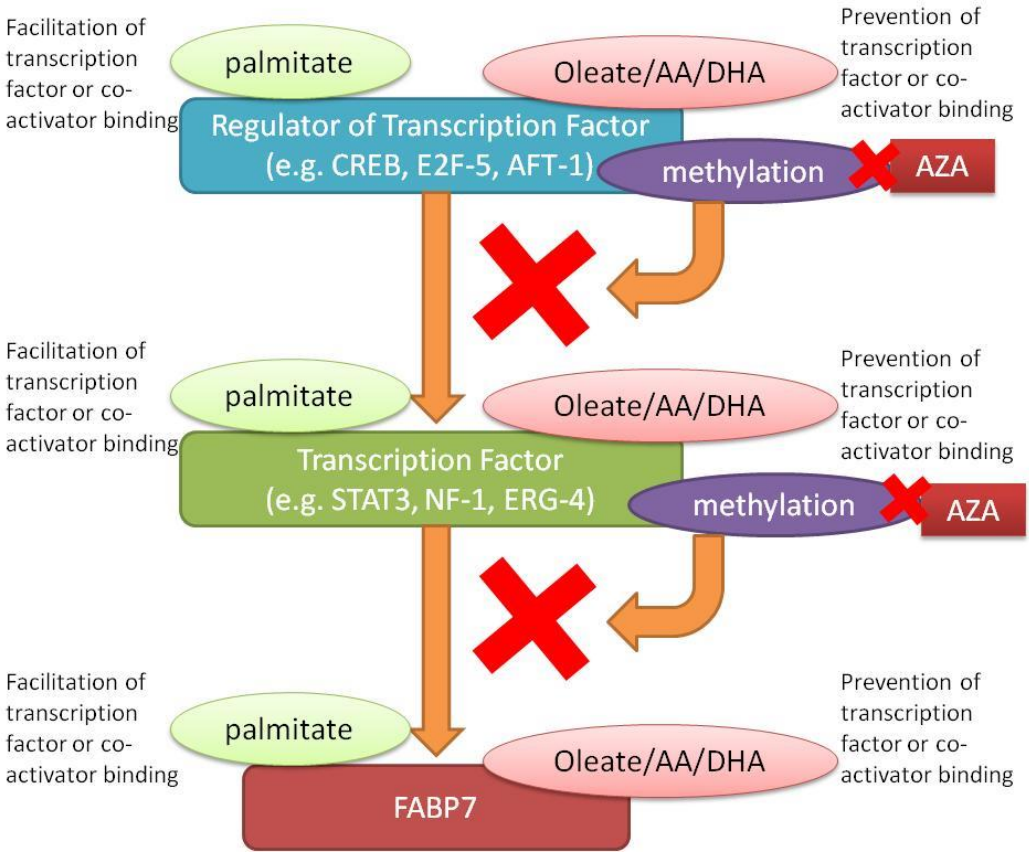


Figure 7.2 The potential mechanisms by which FAs and methylation interact to alter FABP7 expression.

AZA treatment demethylates genes that regulate the expression of FABP7 such as STAT3, NF-1 and ER-4. Additional treatment with palmitate could lead to the facilitation of transcription of FABP7 or genes upstream of FABP7 by increasing transcription or co-activator binding. Oleate, AA and DHA have the opposite effect of palmitate and may prevent binding of transcription factors and co-activators.

Further work would involve identifying transcription factors of FABP7; genecards identifies STAT3 as one potential candidate. A chromatin immunoprecipitation (chIP) assay would be useful in identifying whether STAT3 binds to FABP7 in breast cancer cell lines. If STAT3 is a transcription factor to FABP7 or others were identified, methylation studies could be carried out to further investigate how AZA alters FABP7 expression. An alternative to epigenetic mechanisms and FAs being absent *in vitro* compared to *in vivo*, is the 3D tumour microenvironment. For instance there

is no stromal tissue present within the cell line culture environment; therefore interactions that may stimulate FABP7 expression would not occur. 3D spheroid culture or animal models may help further understand *in vivo* expression of FABP7 and any interaction with stromal tissue.

Over-expression experiments (chapter 5) set out to understand the role of FABP7 and alterations in the phenotype of BT-20 cells, when FABP7 is over-expressed. To summarise, part of the work demonstrated that FABP7 over-expression increased mitochondrial activity/cell viability and reduced cell death. It is unlikely that FABP7 over-expression altered the result of the MTT assay by increasing cell turnover and thus number of mitochondria per well of the culture vessel, because total cell numbers were unchanged between transfection conditions. In relating this finding to the *in vivo* patient sample work (chapter 3), it seems counterintuitive that increased mitochondrial activity/cell viability as a result of FABP7 over-expression relates to FABP7 expression being associated with a better prognosis. If FABP7 positive breast cancer cells *in vivo* were more metabolically active and/or viable, than FABP7 negative counterparts, it seems logical to assume that FABP7 positivity may indicate a poor prognosis. This study has suggested that FABP7 may increase cell viability by decreasing the production of ROS; ROS have been implicated to contribute to tumourigenesis and metastasis by causing damage to mitochondrial DNA and thus disrupting mitochondrial function (Ishikawa *et al.*, 2008). A very recent study has shown that mitochondrial complex I activity is important in regulating breast tumour growth and metastasis through regulation of the balance between NADH and NAD+ (oxidised NADH) and also through generation of ROS (Santidrian *et al.*, 2013). It can be theorised that FABP7 may restore or maintain the energy balance of mitochondrial complex I either by directly making FAs available to the energy pathways or by shuttling the FAs for conversion to molecules that enter the pathway. Alternatively FABP7 may prevent the production of ROS either by preventing or making FAs available to metabolic pathways. In both instances this could explain how FABP7 over-expression

is associated with an improved patient prognosis and increased cell viability/mitochondrial activity. Further work is required to understand the relationship between FABP7 and mitochondrial activity.

FABP7 over-expression did not alter cell death induced by STRP, possibly due to the multiple pathways through which STRP induces apoptosis. This could indicate that FABP7 does not alter sensitivity to cell death. This phenotype may be useful, as chemotherapy drugs used to treat TN breast cancer, induce cell death through a number of pathways. For instance chemotherapy drugs can induce apoptosis through activation of caspases or mitochondrial death pathways, or can induce cell death by necrosis and autophagy (Fulda & Debatin, 2006). This shows that chemotherapy drugs, like STRP, have multiple mechanisms of action; it also could be postulated that FABP7 is not involved with resistance to chemotherapy drugs though further research is required in order to understand FABP7 expression in relation to sensitivity to chemotherapy drugs for TN breast cancer. To investigate the role of FABP7 in sensitivity to cell death, BT-20 cells were treated with FAs post transfection. Relative to the control vector transfected cells, FABP7 over-expression reduced cell death caused by FA treatment. It was postulated that this could be due to FABP7 sequestering the FAs so they are not available to activate apoptotic pathways; for instance palmitate can induce death through conversion to ceramide; FABP7 may bind palmitate and prevent ceramide synthesis so apoptosis is not activated. An alternative is that FABP7 shuttles the FAs to the nucleus so that anti-apoptotic pathways are activated and pro-apoptotic pathways are inhibited. As a result of this theory it is possible that the combination of both FABP7 expression and the intake of various FAs in the diet, play a role in breast cancer development and progression; it would be interesting to investigate further the combined effect of FABP7 and fatty acids on the cell line characteristics, for instance cell migration and invasion. FABP7 over-expression did not alter FA accumulation, as demonstrated with oil red O staining, suggesting that

the role of FABP7 in breast cancer cell lines is to shuttle FAs and solubilise them so that various metabolic and viability pathways are activated or inhibited.

A further aim of the FABP7 over-expression studies (chapter 4) was to investigate whether over-expression of FABP7 altered the expression of bcl-x, survivin and caspase 9 splice variants; these genes are involved in the regulation of apoptosis and survival and the ratio of splice variant expression can give an indication on both anti- and pro- apoptotic properties. There were no changes in the expression of bcl-x splice variants in FABP7 over-expressing BT-20 cells. Bcl-x_L is the variant predominantly over-expressed in breast cancer and is associated with high tumour grade (Olopade *et al.*, 1997). Considering that FABP7 in the present study was associated with a better patient survival, it could be speculated that there would be a reduction in expression of bcl-x_L and/or an increase in the expression of bcl-x_s; however the results indicate that FABP7 does not play a role in bcl-x signalling pathways. There were also no changes in the ratio of caspase 9 splice variants nor any obvious patterns in expression of individual splice variant expression in relation to FABP7 expression. One suggested reason for this was that FABP7 inhibits apoptosis independently of caspase 9; perhaps through inhibition of ROS production in mitochondria or increased mitochondrial activity; this is supported by the MTT assay results in FABP7 over-expressing BT-20 cells. Interestingly there was a trend for FABP7 over-expressing BT-20 cells to have increased expression of survivin ΔEX3, survivin FL and increased ratio of survivin ΔEX3:survivin FL. Although not significant this trend was of note as the increased survivin expression was in line with results showing decreased apoptosis after FA treatment and increased mitochondrial activity/cell viability. It was hypothesised that FABP7 could be shuttling various FAs to the nucleus of cells and alter gene expression, or to the mitochondria to alter mitochondrial activity, energy production and in turn cell viability; in both cases leading to alterations in survivin expression. It is interesting that FABP7 increased survivin expression; as with the increased

mitochondrial activity, it seems counterintuitive that this should be the case considering that in patient samples there is a trend for FABP7 expression to be associated with better survival. There is conflicting evidence as to the role of survivin in breast cancer. For example one study looked at survivin with IHC and found that nuclear staining for survivin was an independent predictor of favourable prognosis (Kennedy *et al.*, 2003). Another study found that survivin was associated with a poor prognosis but nuclear and cytoplasmic localisation was not distinguished (Hinnis *et al.*,; 2007). One study even found that survivin ΔEx3 was correlated positively with apoptosis (Ryan *et al.*, 2005). Further work is required to understand the role of FABP7 in relation to apoptosis and survival and expression of genes in these pathways. A limitation of transient over-expression is that only short term effects of FABP7 over-expression could be studied. Further work could investigate the long term effects of FABP7 expression, possibly using a stable transfection; perhaps trends such as changes in survivin expression may become clearer.

Figure 7.3 was compiled based on the findings of this research. It shows the potential mechanisms that could regulate FABP7 and shows how FABP7 may alter gene expression and cell survival.

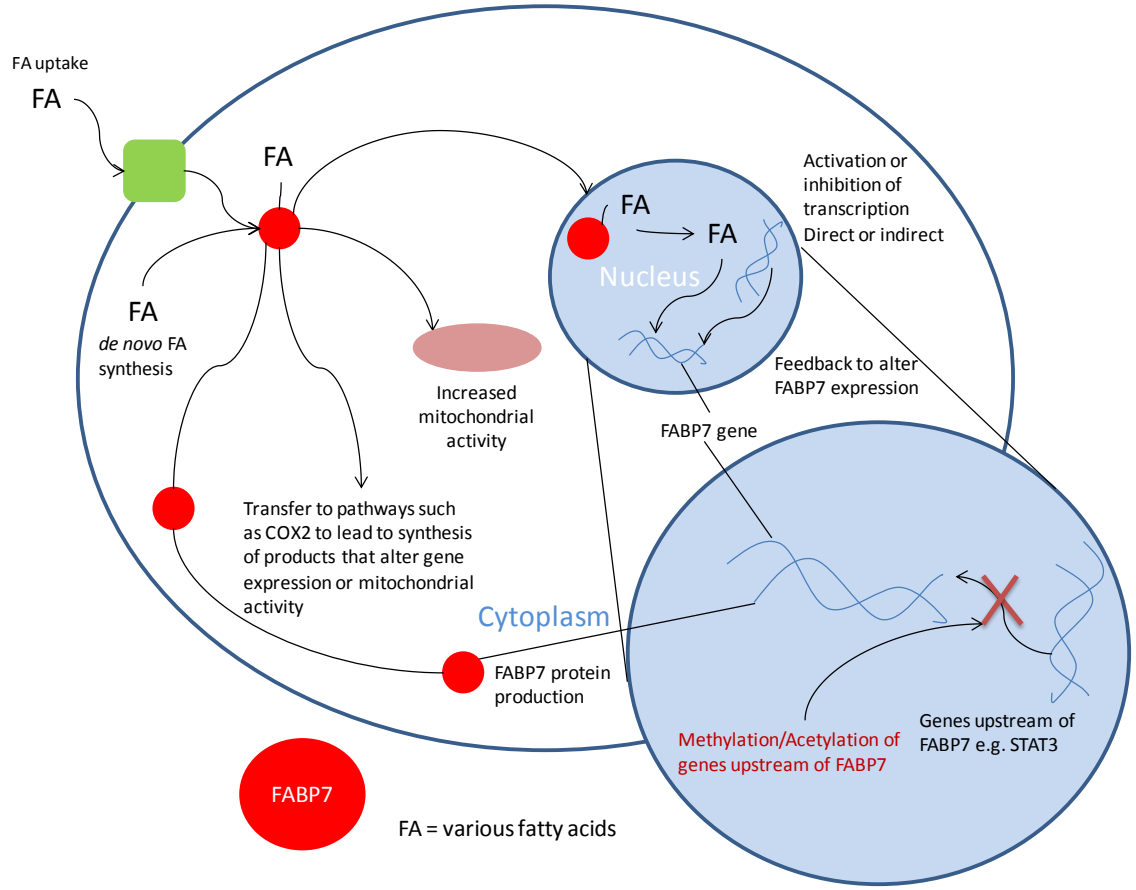


Figure 7.3 The potential roles of FABP7 in TN breast cancer cell lines

This diagram was produced based on the findings of this research. FABP7 is regulated indirectly by methylation, perhaps by the transcription factor STAT3. FABP7 can interact with FAs perhaps and shuttle them to the nucleus. The FAs may then activate or inhibit the transcription of FABP7 directly or indirectly in conjunction with epigenetic mechanisms. FABP7 may shuttle FAs around the cell to impact upon gene expression and mitochondrial activity.

The role of IGFBP-2 and PTEN in TN breast cancer patient samples was investigated (chapter 6). IGFBP-2 is a member of the IGF-axis and is involved in regulating the action and binding of IGFs to the IGF receptor. Similarly to FABP7, IGFBP-2 is involved in aspects of metabolism and breast cancer development and progression. This information is reviewed in section 1.7. IGFBP-2 has actions that are independent of the IGF-axis; figure 1.15 shows that IGFBP-2 down-regulates the

expression of the tumour suppressor gene PTEN in cell line models (Perks *et al.*, 2007). However studies have yet to investigate the expression of IGFBP-2 in TN breast cancer and no study until now has investigated the relationship between IGFBP-2 and PTEN expression in patient samples. The main finding of chapter 6 was that IGFBP-2 expression is significantly associated with loss of PTEN expression in TN breast cancer cases. Loss of PTEN expression was also found to be a frequent event in TN cancer when compared to other studies using the same PTEN antibody for IHC on breast cancer cohorts not selected on the basis of their TN status (Lopez-Knowles *et al.*, 2009). Further studies would investigate whether the relationship found in the current study between IGFBP-2 and PTEN in a Malaysian TN breast cancer cohort, is present in a UK TN cohort and also whether the frequency of PTEN loss found in the present study is comparable to that in a UK TN cohort.

7.1 Conclusions

To summarise, this research has shown that FABP7 expression in patient samples is associated with lower stage of breast cancer, the basal-like phenotype and expression of FAS. There was also a trend for FABP7 positive patient samples to have better survival than FABP7 negative cases. This is the first study to show that FABP7 protein and mRNA is expressed in low but detectable levels in breast cancer cell lines and expression of FABP7 mRNA may be indirectly regulated by epigenetic mechanisms such as methylation. FABP7 over-expression leads to increased mitochondrial activity/cell viability and prevented death after FA treatment. FABP7 over-expression did not significantly alter the expression of bcl-x, survivin and caspase 9 splice variants but there were trends. FABP7 over-expressing BT-20 cells tended to have increased survivin FL and ΔEX3 expression. Both increased mitochondrial activity and survivin expression have been found to be associated with improved prognosis in breast cancer and this may explain some mechanisms by which FABP7 results in better prognosis in the TN breast cancer cases in this

study. This is the first study that demonstrates the significant relationship between IGFBP-2 expression and PTEN loss in patient samples. PTEN loss is a frequent event in TN breast cancer. IGFBP-2 and PTEN loss may be useful markers of prognosis in TN breast cancer.

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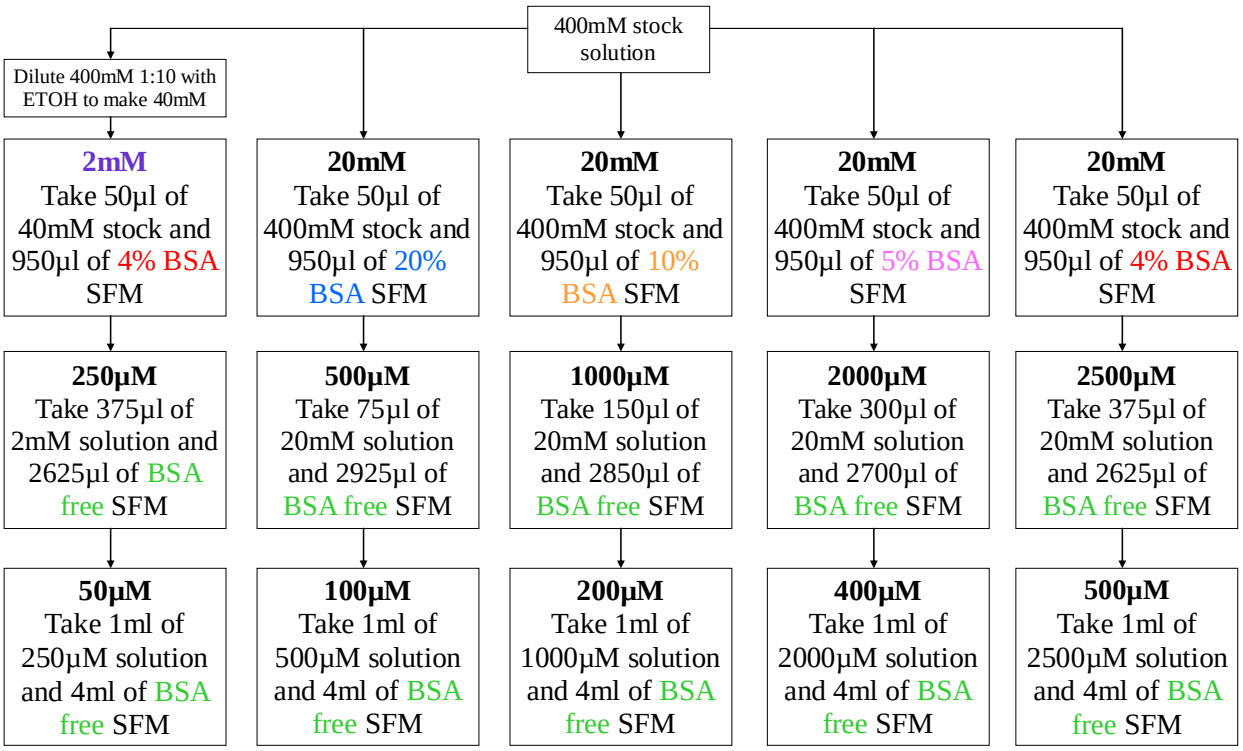
9 Appendix I: Fatty acid dosing method

Oleate and Palmitate Dose Volumes

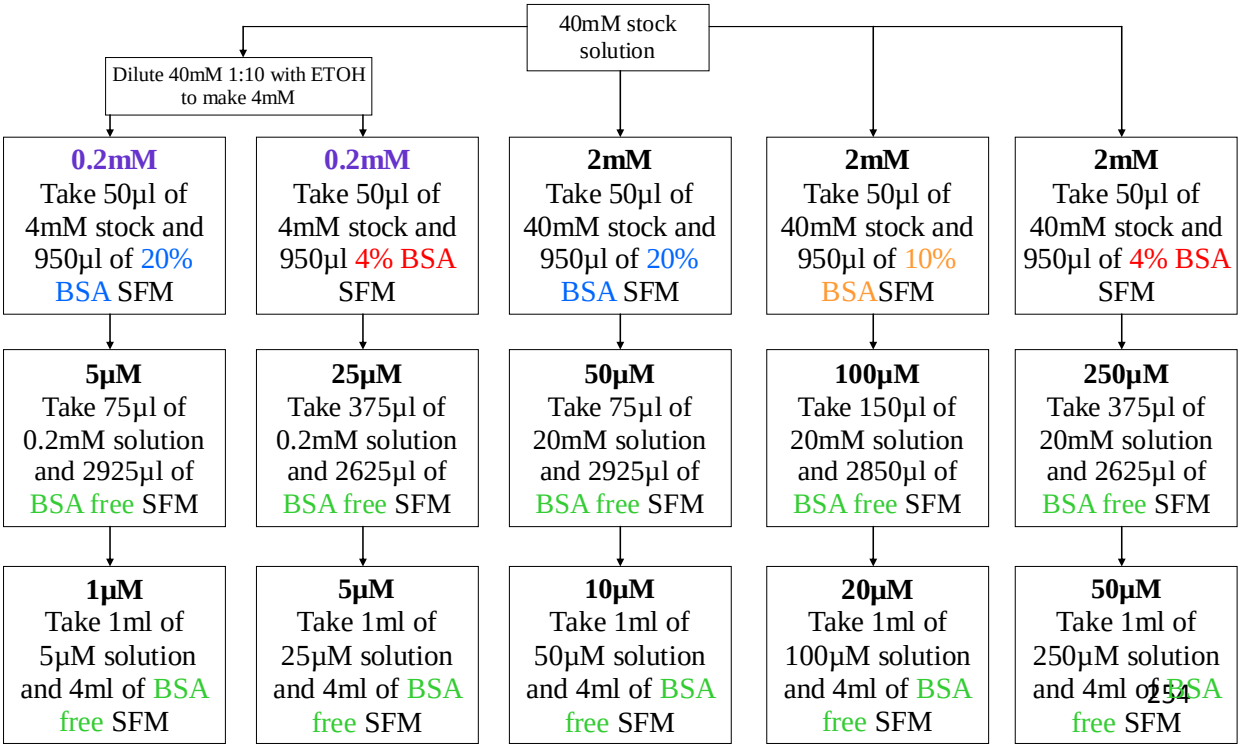


Final concentration of BSA for all doses is 0.1%
Fatty acid free BSA is made up then filter steralised

Final concentration of ETOH for all doses is <0.1%
All solutions are heated and mixed thoroughly at each stage

ETOH is filter steralised

Arachidonic Acid Dose Volumes

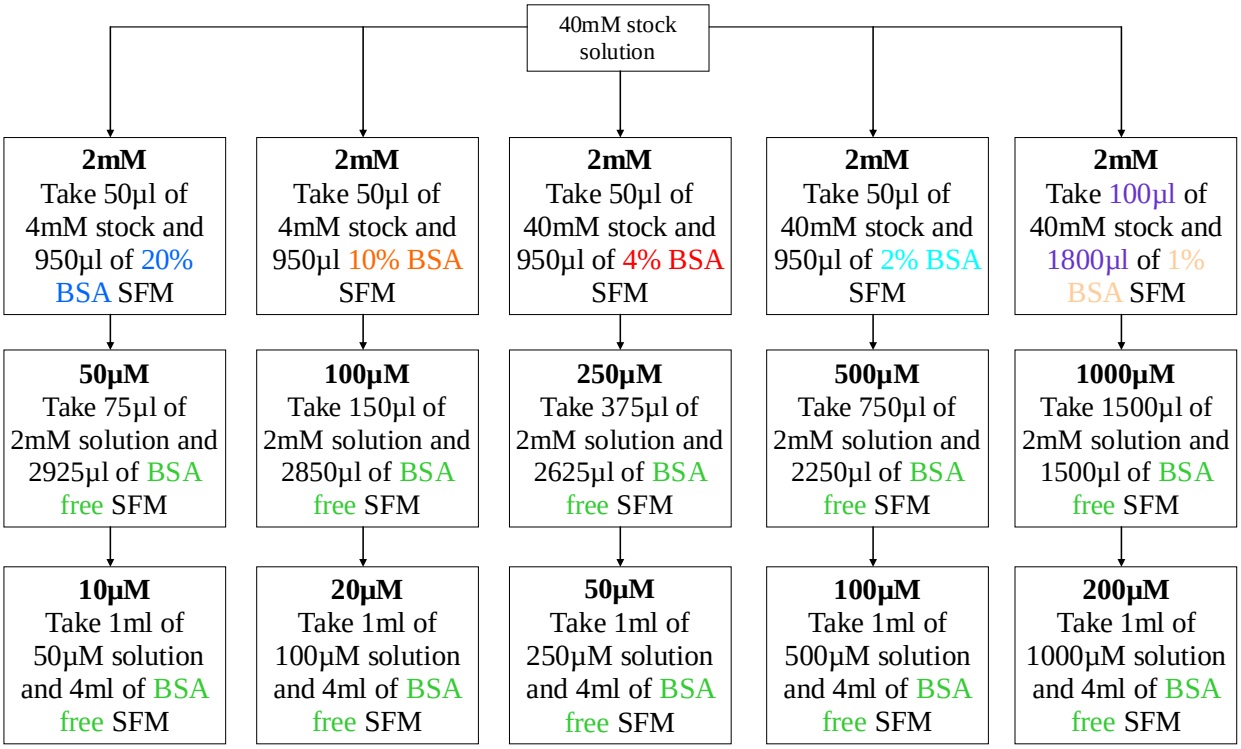


Final concentration of BSA for all doses is 0.1%
Fatty acid free BSA is made up then filter steralised

Final concentration of ETOH for all doses is <0.1%
All solutions are heated and mixed thoroughly at each stage

ETOH is filter steralised

Docosahexaenoic Acid Dose Volumes



Final concentration of BSA for all doses is 0.1%
Fatty acid free BSA is made up then filter steralised

Final concentration of ETOH for all doses is <0.1%
All solutions are heated and mixed thoroughly at each stage

ETOH is filter steralised

10 Publications

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