The Influence and Expression of IGFBP-3 in Normal and Malignant Breast Tissue.

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Abstract

The mitogenic and anti-apoptotic effects of insulin-like growth factor-I (IGF-I) are regulated by a family of insulin-like growth factor binding proteins (IGFBPs), particularly IGFBP-3. *In vitro* studies have demonstrated the importance of the IGF-I axis in regulating the growth of breast cancer cells. Little is known, however, about the IGF-independent role of IGFBP-3 in breast cancer and the mechanisms regulating its production.

We investigated the expression of IGFBP-3 in malignant and paired adjacent normal (n=53), and healthy normal (n=17) breast tissue samples using RT-PCR, immunohistochemistry and ELISA. We compared IGFBP-3 expression with other members of the IGF-I axis, other known tumorigenic genes and clinicopathological parameters. We also developed a novel tissue explant system using fresh normal and malignant breast tissue, with which we examined the *in vitro* effects of IGFBP-3 alone and in combination with known apoptotic agent, doxorubicin (n=6), on tissue viability and apoptosis.

Results demonstrated universal high level of expression of IGFBP-3 mRNA in all types of breast tissue. There was no difference in level of expression between any of the three groups of breast tissue (approaching those seen in the liver where it is predominantly produced). 96% of samples also expressed IGFBP-3 protein. High levels of IGFBP-3 mRNA were associated with the presence of high grade ductal carcinoma-in-situ ($p<0.0001$), the pre-invasive stage in breast cancer. A significant correlation was also found between IGFBP-3 negative/weakly positive tumours and lymph node negativity ($p<0.05$),

thereby supporting an association between IGFBP-3 and the development of invasive disease. There was, however, no siginificant correlation between IGFBP-3 expression and other clinicopathological parameters.

The *in vitro* tissue explant system demonstrated that IGFBP-3 had little effect by itself on apoptosis. However, when used in combination with doxorubicin, a marked enhancement of apoptosis was seen in breast tumours. In contrast, less apoptosis was seen in normal breast tissue suggesting a protective effect. These divergent effects suggest a potential novel chemotherapeutic approach in the treatment of breast cancer.

These findings suggest that IGFBP-3 may play a role in tumorigenesis. It may therefore be possible in future, that IGFBP-3 levels could be used in cancer risk assessment and prevention or infact, as markers of response to cancer treatments.

Table of contents

List of Figures

List of Tables

1. Introduction

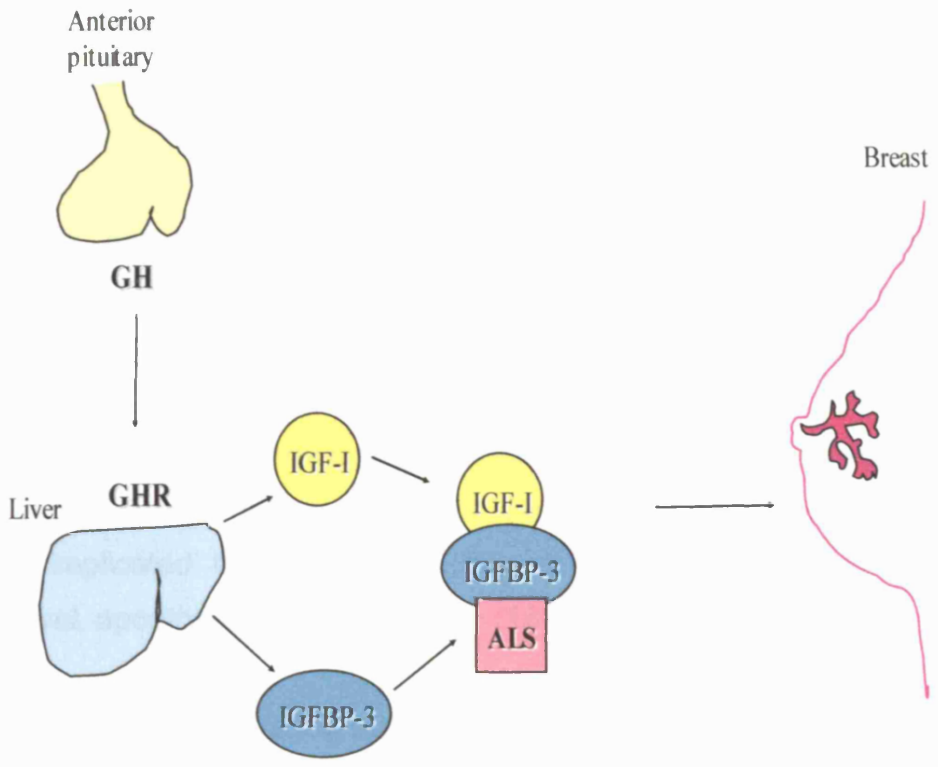
1.1 The GH-IGF-I axis

The growth hormone-IGF-I axis has been increasingly implicated in the development of cancer. It has been known for some time that patients with acromegaly who have increased levels of growth hormone (GH) and IGF-I, are at increased risk of colon cancer and also have increased mortality due to other malignant and vascular diseases (1;2). IGF-I was discovered more than 40 years ago and is known to be a mediator of growth hormone (GH) activity. GH is essential for linear growth and development and circulating levels decline progressively with age. Serum IGF-I levels similarly decline with age and largely reflect GH secretion and therefore have been used clinically as a marker of GH status.

IGF-I is present at high levels throughout the body. It circulates at concentrations approximately 1000 times higher than most peptide hormones. GH is considered to be the predominant regulator of circulating IGF-I, however, there are other factors that contribute to IGF-I concentrations in serum and tissue. Age, sex and nutritional status all affect IGF-I levels (3). Other factors include insulin-like growth factor binding protein-3 (IGFBP-3) which binds approximately 85% of IGF-I and maintains its high serum concentration by slowing it's clearance. Other binding proteins bind approximately 15% of IGF-I and the rest of IGF-I circulates free (4). IGF-I binding to the IGF-IR may prevent apoptosis and the presence of IGF-IR on some tumours can implicate a more aggressive prognosis (5;6). At a tissue level, IGF-I is an important regulator of cell survival, growth and metabolism.

Insulin-like growth factor binding protein-3 (IGFBP-3) is a 40-44kDa glycoprotein of hepatic origin and is responsible for carrying approximately 90% of circulating IGF in the serum. Unlike IGF-I, IGFBP-3 levels are relatively stable and are less susceptible to nutritional effects (7). It exists as a 150-kDa ternary complex consisting of IGF-I, IGFBP-3 and a bound acid labile subunit (ALS). The large size of this ternary complex prevents it from crossing the capillary barrier (8) and also prolongs its half-life from 10 minutes in the unbound state, to approximately 12 hours. IGFBP-3 may therefore inhibit the mitogenic actions of IGF-I by binding to it with such affinity that it prevents IGF-I from binding to its own receptor (Fig.1). It may also enhance its effects by increasing its bioavailability at the target site (9-11). Within the last decade it has become clear that IGFBP-3 could inhibit cell growth and induce apoptosis by IGF- independent mechanisms. Little is known, however, about the IGF-independent role of IGFBP-3 in cancer and the mechanisms regulating its production.

Figure 1. Summary of growth hormone- IGF axis demonstrating the production of IGF-I and IGFBP-3 from the liver and the formation of the ternary complex



GH = growth hormone

GHR = growth hormone receptor

ALS = acid labile subunit

IGF-I = insulin-like growth factor-1

IGFBP-3 = insulin-like growth factor binding protein-3

IGFBP-3 is one of a family of six high affinity IGF-binding proteins present in the circulation (IGFBP-1 – 6) (12) which modulate the interactions between IGFs I and II and their receptors. There are also several low affinity binding proteins that have recently been named IGFBP-related proteins (IGFBP-rPs). IGFBPs have been shown to be produced in almost all tissues to varying degrees, however, IGFBP-3, the most abundant IGFBP, is produced predominantly from the liver (13).

IGFBPs were originally described as passive circulating transport proteins for IGFs. However they are also implicated in a complex variety of cellular functions, and exert actions also at a tissue level, depending on their primary structure and their post-translational modifications. IGFBPs also have important IGF independent endocrine and paracrine effects as well as their well documented effects on IGF-I (14-16). Previous studies have also implicated the role of IGFBPs in cell motility, adhesion(12;17), survival, apoptosis and the cell cycle (18-20).

IGFBPs are known to interact with several signalling pathways such as PI3-kinase pathway (21), the type II transforming growth factor-β (22) and the retinoid-X receptor (23). They may also have a unique signalling pathway of their own.

IGFBP-3 is the main circulating binding protein in serum and circulates as a large ternary complex (150kDa). It controls the bioavailibity of IGF-I by prolonging its half-life. Recent studies have shown that IGFBP-5 is also capable of forming a ternary complex with IGF-I or II and the ALS (24) which is slightly smaller in size (130kDa). It is therefore possible for IGF-I to be transported around the circulation in the absence of IGFBP-3. Small amounts of IGF-I bind to IGF binding proteins as a binary complex and less than 1% travel free in the circulation (8).

The main function of IGFBP-3 was thought to be as a 'reservoir' for IGF-I. In serum, the IGF/BP-3 ternary complex is present in ten-fold concentrations greater than all other binding proteins (25). IGFBP-3 also has a higher affinity for IGF-I than all the other binding proteins and similarly, IGF-I is thought to bind with higher affinity to IGFBP-3 than it does to IGF-IR (26).

The ALS component of the ternary complex also has an important role in modulating IGF-I and IGFBPs. (27). The normal half-life of free IGF-I is approximately 10 minutes and that of IGFBP-3 is 30-90 minutes. The half-life of the ternary complex, however, is 12 hours. Therefore it serves to greatly prolong the half-life of IGF-I (28). IGFBP proteases (IGFBP-Pr) also play an important role in modulating levels of IGF-I as they cleave IGFBPs. Free IGF-I is then released and able to exert its effects at a local level.

1.1.1 IGF dependent effects

It is well known that IGF-I binds to IGFBP-3 with greater affinity than it does to its own receptor and this therefore enables it to tightly control tissue activity (29). IGFBP-3 may be either inhibitory or stimulatory to IGF actions (30;31). The high affinity with which IGFBP-3 binds to IGF-I is thought to have an inhibitory effect on the action of IGF-I, by preventing it from interacting with the IGFI-R (9-11). One study investigating this used IGF-I analog des-IGF-I which binds and activates IGF-IR but does not bind BP-3. In the human HL-60 promyeloid cell line, BP-3 inhibited the proliferative effects of IGF-I and –II but did not inhibit the proliferative effects of des-IGF-I. The anti-proliferative effects of BP-s, therefore, may be dependent on the binding and sequestration of IGF-I (32). Conversely, IGFBP-3 may also enhance the action of IGF-I by providing a reservoir of IGF-I and increasing the amount of IGF-I at a local tissue level that once

free, is able to exert its effects. In the breast, IGFBP-3 is thought to have a predominantly inhibitory effect. Interestingly, it has also been shown to induce apoptosis by sequestering IGF-I in MCF-7 cells (33).

1.1.2 IGF independent effects

It is becoming increasingly clear that as well as modulating the activity of IGF-I, IGFBP-3 exerts its own effects. The IGF-I independent pro-apoptotic effects of IGFBP-3 were first demonstrated in 1993 by Rosenfeld *et al* when it was observed that IGFBP-3 inhibited the growth of Hs578T breast cancer cells which do not respond to IGF-I (34). Rajah *et al* also observed in 1997 that when IGFBP-3 was added to an IGF-I negative mouse fibroblast cell line, a significant increase in apoptosis was observed (18). Valentinis *et al* used fibroblasts from IGF-IR knockout mice which demonstrated growth inhibition when transfected with a vector containing the IGFBP-3 gene (35). This growth inhibitory effect of BP-3 was therefore mediated by an IGF-independent pathway.

Studies performed by Perks *et al* looked at the apoptotic effects of IGFBP-3 on Hs578T cells and found that when used on its own, IGFBP-3 had minimal effects, however, when used with a ceramide analogue (C2), apoptosis was significantly accentuated (36;37). Furthermore, where IGFBP-3 significantly accentuated this apoptosis, IGFBP-4 and -5 reduced C2-induced apoptosis and IGFBP-1, -2 and -6 had no significant effect at all. This suggests that the IGFBP family exerts its apoptotic effects through differing apoptotic signalling pathways.

Other studies looking at the IGFBP-3 enhancement of C2-induced apoptosis in Hs578T cells have also suggested a possible link with cell association and binding protein proteolysis (38), an effect which may be reversed when treated with IGF-I. As well as ceramide analogue, C2, IGFBP-3 has also been shown to significantly accentuate the apoptosis

caused by DNA-damaging stimuli such as ionizing and UV irradiation and chemotherapeutic drugs such as paclitaxel (39-42).

Some studies have demonstrated that IGFBP-3 is capable of inducing apoptosis on its own as well as potentiating the apoptotic effects of other DNA damaging agents such as UV radiation and cytotoxics (39-41). Further transfection of IGFBP-3 into radiation resistant T47D breast cancer cells restored the sensitivity to ionising radiation. This is one of the only studies demonstrating an independent apoptotic effect of IGFBP-3 ; mostly IGFBP-3 has been shown to enhance the apoptotic effect of other agents. Cell culture studies are, however, difficult to assess as some degree of apoptosis always occurs and in general, IGFBP-3 shows a more marked effect when used in combination with other apoptotic agents.

A study performed in 1995 reported that IGFBP-3 gene expression was induced by tumour suppressor gene p53 (43). In colon cancer cells, IGFBP-3 was shown to be a mediator of p53-dependent apoptosis (41;43) and similarly in an oesophageal cancer cell line, apoptosis after prior irradiation demonstrated an increase in p53 which was further enhanced by addition of exogenous IGFBP-3 (41). Butt *et al* transfected IGFBP-3 into wild-type (MCF-7) and mutant (T47D) breast cancer cells resulting in apoptosis, thereby suggesting that apoptosis may occur independently of functional p53 (22;40). Apoptosis seen in breast cancer cells over expressing IGFBP-3 has been shown to be associated with an increase in Bad and Bax, which are pro-apoptotic, and a decrease in Bcl-2 and Bcl-x_L, which are anti-apoptotic (40). This suggests that IGFBP-3 may cause apoptosis using this apoptotic signalling pathway. Similarly in studies looking at apoptosis in a prostate cancer cell line (PC-3), apoptosis induced by IGFBP-3 was inhibited by a caspase inhibitor(18). Caspases are a family of cysteine aspartic acid-specific proteases which play a central role in the apoptotic pathway (44).

The mechanisms underlying these IGF independent effects have yet to be elucidated. Unfortunately, a specific IGFBP-3 receptor has not been fully discovered (34;45).Oh *et al* suggested that a cell surface IGFBP-3 receptor may exist in 1993. In this study, the inhibitory effects of oestrogen receptor negative Hs578T breast cancer cells were shown to be dose dependent and diminished by co-incubation with IGFs, but not by analogs with reduced affinity for BP-3 (46). A putative receptor has been cloned and initial data shows direct activation of caspase 8 in response to BP-3 binding. Over expression of this receptor has been shown to increase cell surface association of IGFBP-3 and apoptosis, however, further work is needed to fully assess the mechanism of action (23).

1.2 IGFBP structure

The IGFBP genes share a common structural relationship whereby four conserved exons are located within genes ranging from 5kb (IGFBP-1) to greater than 30kb (IGFBP-2 and IGFBP-5) (47).

All six binding proteins are well conserved and have been shown to share an overall protein sequence homology of 50% within a species (48;49). The most highly conserved regions include the N-terminal ligand-binding domain and the C-terminal region which is thought to be involved in protein and extracellular matrix (ECM) interactions. There are also many conserved cysteine residues within these regions. These residues are thought to be important in forming the high affinity bonds with IGF-I and IGFBPs that lack them have a reduced ability to bind IGF-I and II (50). Previous studies have shown that IGFBPs from fish, reptiles and birds are able to specifically bind ^{125}I-labeled mammalian IGF-I (51;52), thereby providing evidence that IGFBPs were present in early vertebrate evolution despite the fact that no IGFBP genes have been cloned in this group.

The structural differences between the IGFBPs may account for their differential actions (29). The RGD sequence (arginine-glycine-aspartic acid) is present in IGFBP-1 and -2 and is thought to facilitate the association of IGFBP-1 with cell surface by the fibronectin receptor (12). All IGFBPs contain heparin binding sites which enable the attachment to cell surface/ECM (53). IGFBP-3 and -5 demonstrate the highest affinity of heparin binding sites and also possess nuclear localisation sequences (54); structurally, they are the most similar. IGFBP-3 is known to be N-glycosylated (55) and IGFBP-5 is known to be O-glycosylated (56) which are associated with cell and matrix association, although studies have shown that glycosylation of IGFBP-3 has no significant effect on IGF-I (57) or ALS binding (58). The significance of these post-translation modifications is not fully understood. However, they may contribute to the prolonged half life of IGFBP-3 in the circulation.

1.2.1 IGFBP proteases

IGFBP proteases were first described in the circulation of pregnant women (59-61). IGFBP proteases have been shown to cleave IGFBP-2 to 6 with the production of fragments that have a greatly reduced affinity for IGF-I. This cleavage therefore means that more IGF-I is made freely available to exert its effects via the IGF-IR. Since then, an increase in protease activity has been reported in several pathophysiological conditions, such as diabetes and severe illness, leading to the possibility that they may be autocrine/paracrine growth regulators (62-64). An increased protease activity has also been reported in advanced breast cancer and other advanced malignancies (65;66). It was proposed that an increased protease activity may be implicated in breast tumorigenesis by facilitating the release of IGF-I at a tissue level (67).

Proteases implicated in the specific cleavage of IGFBP-3 include prostate-specific antigen (PSA), cathepsin D and plasmin. All have been demonstrated in breast cancers. PSA, usually found in seminal fluid, has recently been reported to be synthesized by the breast (68-70). Measurement of PSA levels and IGFBP-3 has been found to have significant prognostic implications in screening for prostate cancer (71). Reports, however, have been conflicting with high levels of PSA reported in benign disease and lower levels in advanced cancer (68). It has also been reported in a study of 200 breast tumour cytosols, that no correlation exists between PSA and IGFBP-3 levels (expression) (70).

Plasmin has also been reported to be a prognostic marker in breast cancer (72). It is thought to proteolyse IGFBP-3 into fragments which are able to inhibit the mitogenic effects of IGF-I despite having a reduced affinity for IGF-I (73). This is further support for IGFBP-3 independent effects since it suggests that IGFBP-3 fragments are independently bioactive.

High levels of cathepsin D have been significantly correlated with the development of metastases in breast cancer in one study that measured levels in the cytosol of breast tumours (74;75). It has also been reported that oestrogen enhances the secretion of cathepsin D in oestrogen receptor postitive and not negative breast cancer cells (76;77).

A high level of proteolytic activity is generally thought to result in an increased risk of tumourigenesis since IGF-I is cleaved from IGFBP-3 and free to exert its effects. There is some evidence, however, that the fragments produced by IGFBP-3 cleavage may also exert anti-proliferative effects on breast cancer cells. The exact role of IGFBP proteases, therefore, have yet to be fully elucidated.

1.2.2 IGFBP-related proteins (IGFBP-rP)

IGFBP-rPs are a group of newly identified proteins which share some homology with the amino-terminal end of IGFBPs (78). There appears to be some structural differences in the carboxy-terminal end of IGFBP-rPs, thereby accounting for the reduced affinity for which they bind IGF-I. Due to this reduced affinity binding, their role in the bioactivity of IGF-I is not thought to be physiological. IGFBP-rP-1 does in fact bind insulin with high affinity (79), thereby suggesting an IGF independent function.

IGFBP-rP1 has been found to be over expressed in normal breast epithelial cells and down regulated in breast tumours (80). Similarly it has been found to be down regulated in prostate cancer and is thought to be a potential tumour suppressor protein for prostate cancer (81).

1.3 GH-IGF-I axis and breast cancer

There are various stages at which IGF-I and IGFBP-3 are thought to contribute to the development of cancer. IGF-I may directly influence the initial stages of malignant transformation by enhancing cell survival, migration and proliferation. Similarly, it may interfere with the normal mechanisms of cell cycle arrest and apoptosis. It may also play a part in the development of local invasion and metastases and later on, in the development of resistance to therapeutic agents. In addition to these direct effects, IGF-I may indirectly promote tumourigenesis by interacting with oncogenes, tumour suppressor genes, other hormones, such as oestrogen in breast cancer and IGF binding proteins, particularly IGFBP-3. It also induces vascular endothelial growth factor (VEGF) which has well known angiogenic effects.

IGFBP-3 may indirectly influence tumorigenesis by binding IGF-I and thereby inhibiting the mitogenic effects of IGF-I. It may also directly effect tumourigenesis by it's own anti-proliferative and pro-apoptotic effects. In this way, IGFBP-3 is thought to play a cancer protective role. Normal tissue homeostasis is dependent on the tight control of cellular proliferation and apoptosis. Any abnormality in this regulation may result in an increase in cell number or survival of cells which have acquired mutations. The mechanisms by which BP-3 may alter cancer susceptibility, however, remains undetermined.

IGF-I is a potent mitogen and has marked anti-apoptotic effects on normal and malignant breast cancer cells(12;82). In mice the development of the breast requires IGF-I action (83;84). Transgenic mice that over express genes encoding GH or IGF-I receptor (IGF-IR) also have an increased incidence of mammary gland epithelial hyperplasia and an increased risk of breast cancers (85;86). IGF-I exerts its effects on cellular proliferation and apoptosis via the IGF-IR which is a trans-membrane tyrosine kinase receptor and over expressed in breast cancer tissue (87). A negative feedback loop exists whereby free IGF-I suppresses the secretion of GH from the pituitary (88). IGF-I also regulates the expression of several genes associated with breast tumourigenesis; c-myc, VEGF, progesterone receptor (PgR) and cathepsin D (89). In the absence of IGF-I action, several known oncogenes are unable to produce malignant transformation, however, with re-expression of the IGF-IR, the malignant potential of these oncogenes is restored (90-95).

1.3.1 Epidemiological studies

Epidemiological studies on IGFBP-3 and breast cancer are controversial. Some studies have shown a link between a high serum IGF-I and a low

serum IGFBP-3 with an increased risk of developing breast cancer in pre- and post-menopausal women (96-98). A few studies, however, have failed to demonstrate an association (96;99;100). Recent studies have shown that high serum levels of IGF-I and low IGFBP-3 were present in women with stage I and II breast cancer (101). A high ratio IGF-I/IGFBP-3 ratio was therefore associated with an increased risk of developing breast cancer. Another study reported a link between a high serum IGF-I and low IGFBP-3 with an increased risk of pre-menopausal ductal carcinoma in situ (102). It has been thought that it is not the absolute serum level of IGF-I or BP-3 that is important, more the molar ratio between them. Therefore having a high IGF- I to a low BP-3 ratio would imply an increased risk of developing breast cancer and conversely a low IGF-I to a high BP3 would protect against cancer.

A recent prospective population study has provided evidence that a high serum IGF-I to a low BP-3 ratio is associated with a high risk of developing breast cancer in pre-menopausal women (96). Other epidemiological studies have shown a similar link with colon, prostate and lung cancer (103-105). These studies demonstrate that serum IGF-I is positively associated with risk while IGFBP-3 is negatively associated. The overall increase in relative risk (2-4 fold) appears to be small when compared to the increase in risk associated genetic mutations such as BRCA1/2, however, such mutations are rare. It seems therefore that a high IGF-I/BP-3 ratio is likely to be more common in the population and so the overall increase in cancer risk may be significant.

Interestingly, a recent meta-analysis of 20 studies compared uppermost and lowermost centiles of IGFBP-3 serum levels and reported that high serum levels of IGFBP-3 are associated with a significant increase in the risk of pre-menopausal breast cancer (98). This study also showed that increasing IGF-I concentrations were associated with an increased risk of pre-menopausal breast cancer. No protective effects of higher concentrations of BP-3 were detected. Another recent study looking at

the IGF axis and breast cancer in Chinese women similarly found that IGFBP-3 and IGF-I levels were higher in women with breast cancer versus controls (106). The adjusted odds ratio was 3.01 for women in the highest IGFBP-3 tertile when compared to those in the lowest tertile, thereby supporting the previous meta-analysis' findings of an association between breast cancer and a high BP-3. In some culture conditions, IGFBP-3 has been shown to have anti-apoptotic effects (19;107), possibly reflecting an acquired resistance to its usual growth inhibitory effects and may account for the unexpected positive associations between BP-3 and breast cancer in the above two studies.

In studies looking at other cancers, a positive correlation was seen between high IGF-I/low BP-3 and subsequent risk of developing prostate, colorectal and lung cancer (108-110). The meta-analysis performed by Renehan *et al*, however, reported that IGF-I levels in the upper versus lower tertile were associated with an increased risk of prostate, colorectal cancer and pre-menopausal breast cancer only. They reported that no inverse associations with IGFBP-3 were detected, except for lung cancer (98).

These studies provide evidence for an association between IGFBP-3 and breast cancer risk but do not establish causality. It remains to be determined what the biological significance is for different levels of IGFBP-3 and what controls local expression resulting in tumourigenesis.

1.3.2 In Vitro and animal studies

IGFBP-3 has multiple, complex actions in different tissues. It has been shown to inhibit or potentiate the actions of IGF-I in different culture systems (111;112). A study looking at a prostate cancer cell line (PC-3) demonstrated a growth-stimulatory effect that was thought to be dependent on the active proteolysis of IGFBP-3 via prostate specific

antigen (PSA, a known IGFBP-3 protease) (113). Two other studies have shown anti-apoptotic effects (107;110). Most studies, however, have demonstrated that IGFBP-3 has an inhibitory effect on breast cancer cell growth. Oh *et al* demonstrated that IGFBP-3 inhibited growth of Hs578T cells independent of IGF-I (34). Gill *et al* also demonstrated similar effects in this cell line and also showed that with the use of known apoptotic ceramide analogue, C2, apoptosis could be further accentuated (36). Similarly, IGFBP-3 has been shown to enhance the apoptotic effect of known cytotoxic, paclitaxel which is a drug that works via TNFα and ceramide generation (114).

IGFBP-3 markedly inhibits growth of breast cancer cell line MCF-10A (115) and also in the MCF-7 cell line (33). In a study looking at the effects of IGFBP-3 in T47D cells, growth inhibitory effects were initially observed, however, after several passages cells became resistant to these inhibitory effects and proliferation was seen (19). It was proposed that the development of resistance to IGFBP-3's inhibitory effects may play an important role in the progression of breast cancer. Interestingly, IGFBP-1, 2, 4, 5 and 6 have not been shown to have any growth inhibitory effects on breast cancer cell line, Hs578T (37).

Previous *in vitro* studies have shown a link between IGFBP expression and oestrogen receptor status in breast cancer cells. IGFBP-3 and 1 are found in oestrogen receptor negative cell lines and IGFBP-2 is predominantly found in oestrogen receptor positive cells (116-118). Estradiol has been shown to decrease production of IGFBP-3 in MCF-7 cells (33) and other studies have confirmed a marked increase in IGFBP-3 mRNA in oestrogen receptor negative tumours (119-121).

Early animal studies showed that IGFBP-3 inhibited DNA synthesis in chick embryo fibroblasts when stimulated by serum, fibroblast growth factor and TGFβ (122;123). Further studies with mouse fibroblasts

showed that IGFBP-3 had a growth inhibitory effect in serum-containing media, an effect which was not reversed by insulin (124). This lead to the idea that the growth inhibitory effect of IGFBP-3 was IGF-independent. Fibroblasts from IGF-IR knockout mice were then transfected with IGFBP-3 resulting in growth inhibition and it was thought that IGFBP-3 may be acting by blocking a different receptor (35). Lee *et al* stably transfected non-small cell lung cancer (NSCLC) cell line NCI-H23 with IGFBP-3 cDNA and demonstrated that xenotransplantation into nude mice resulted in minimal tumour growth (125). In another study, adenoviral IGFBP-3 construct was injected into H12999 NSCLC xenografts and a massive destruction of tumours was observed (126).

One study implanted prostate cancer xenografts into SCID mice and the animals were subsequently treated with IGFBP-3 and retinoid-X-receptor (RXR) ligands (23). When used alone, no effect was seen on tumour growth, however, in combination a marked reduction in tumour size was observed. A similar reduction in tumour size was reported when IGFBP-3 was given to SCID mice implanted with colon cancer (127).

Other knockout mice studies for all members of the IGFBP family have only demonstrated minor changes in phenotype suggesting that there may be compensatory changes in other members of the IGFBP family. Mice overexpressing IGFBP-3 resulted in selective organomegaly and mammary gland-targeted expression of IGFBP-3 resulted in an altered involution after pregnancy (128). These findings demonstrate that local action of IGF-I may be altered and that there may be an IGF independent effect locally on tissue growth and development.

1.3.3 The IGF axis and oestrogen signalling

The IGF-axis and oestrogen appear to have a profound synergistic effect on breast cancer cells. Previous mice studies have shown that the

mammary gland needs IGF-I and oestrogen in order to develop fully and without IGF-I, full development does not occur (83;84). *In vitro* studies have confirmed that co-treatment of MCF-7 cells with IGF-I and oestrogen results in proliferation and this effect is blocked by anti-IGF-I antibodies (129). Other studies have shown that in the absence of oestrogen, IGF-I still activates oestrogen receptor-mediated gene transcription and that the synergistic effect of both IGF-I and oestrogen results in an enhanced transcription of oestrogen receptor regulated genes (130).

Oestrogen is also known to act at several points within the IGF-I signal transduction pathways. When IGF-I binds to IGFI-R, auto phosphorylation of the receptor β subunits occurs with a resultant increase in receptor tyrosine kinase activity (129). This leads to phosphorylation of IRS-I (insulin-receptor substrate protein-1) and activation of other downstream signalling pathways involved in mediating mitogenic responses such as phosphatidylinositol 3-phosphate kinase (PI-3K) and C-Jun N-terminal kinase (131-133). Similarly, oestrogen activates several components of the MAP kinase pathway (134)and also acts synergistically with IGF-I to enhance Akt protein synthesis and activity (135).

This increase in Akt protein synthesis and activity results in the phosphorylation of BAD, an apoptosis inducing member of the Bcl-2 protein family. The result is that BAD is sequestered and so unable to bind and inactivate anti-apoptotic Bcl-2 and Bcl-XI thereby resulting in the suppression of apoptosis. One study has shown that breast cancer cells are protected from chemotherapy induced apoptosis by IGF-I and that this effect is mediated by PI-3K (136).

In the clinical treatment of breast cancer, Tamoxifen is used to block the effects of oestrogen. Studies have shown that as well as blocking oestrogen, it also suppresses serum IGF-I levels, probably as a result of suppression of GH secretion (137;138). *In vitro* studies have shown that Tamoxifen also blocks IGF-I mediated proliferation of breast cancer cells.

In addition to these effects, Tamoxifen has also been reported to increase IGFBP-3 levels and thereby reduce the amount of bioactive IGF-I available (139).

In breast cancer tissue studies, estradiol has been shown to increase mRNA expression of IGF-I four to six fold in normal breast tissue (83). It has also been shown to up regulate expression of IGFI-R mRNA expression in oestrogen receptor (ER) positive breast cancer cells (140). Estradiol further increases IGF-I bioactivity by down regulating IGFBP-3 synthesis and increasing synthesis of cathespin D, an IGFBP-3 protease which acts to degrade IGFBP-3 (141).

In contrast to these effects, progestin appears to inhibit oestrogen stimulated growth in ER-positive breast cancer cells (142) and progesterone agonists in fact down regulate IGFI-R mRNA in progesterone receptor (PR) positive breast cancer cells. A down regulation of IGFBP production was also seen in this study (143).

1.4 Expression in breast tissue

Most studies looking at the role of the IGF axis in breast cancer have concentrated on *in vitro* studies using breast cancer cell lines. There has, however, become increasing *in vivo* evidence of a role for the IGF system in breast cancer tissues.

Interestingly, IGF-I mRNA has been detected in normal and malignant breast tissue (144), whereas it does not appear to be expressed from several breast cancer cell lines (145). Another study has demonstrated that stromal fibroblasts rather than epithelial cells are the main source of the local protein, supporting the role of IGF-I acting in a paracrine manner (146). IGF-I mRNA expression has also been found to correlate positively with degree of tumour differentiation (147) and negatively with vascular

invasion (148). Both IGF-I and IGF-IR mRNA have both been found to correlate positively with oestrogen (ER) and progesterone (PgR) status (149) and since a strong ER/PgR status is associated with an improved prognosis, they may carry some additional prognostic information (150).

Several IGFBPs have been found to be expressed by various breast cancer cell lines, however, it does appear to correlate with hormone receptor status (151).Cell lines MDA-330, MDA-231 and Hs578T are hormone receptor negative and express higher levels of IGFBP-3, whereas hormone receptor positive cells MCF-7, T-47-D and BT-20 express higher levels of IGFBP-2 and -4 (117). These cell line studies, however, may not truely reflect physiologically relevant conditions. Similarly, IGFBPs appear to be over expressed in breast cancer tissue when compared to normal breast tissue and their expression differs according to hormone receptor status as it does in breast cancer cell lines. IGFBP-3 mRNA therefore appears to be higher in hormone receptor negative breast tumours (152). Tumour levels of IGFBP-3 have also been positively correlated with tumour size (153).IGFBP 1-5 have been detected in *in vivo* breast cancer specimens, however, whether it is produced by the actual tumour cells themselves or the surrounding stroma has never been documented (120). These few *in vivo* studies, however, have small sample sizes and therefore definitive IGFBP expression in breast tissue has yet to be elucidated.

1.5 Local versus endocrine effects

The biological significance of locally produced versus systemically delivered IGF-I and BP-3 is yet to be elucidated. It is thought that there may be several mechanisms by which IGF-I enhances the mitogenicity of breast cancer cells. Firstly, IGF-I may stimulate the proliferation of breast cancer cells in an endocrine fashion (128) and secondly, the surrounding stroma may produce IGF-I which may produce mitogenic effects in a paracrine or autocrine fashion (145).

A study involving IGF-I knockout mice with a reduction of plasma IGF-I by 25%, did not demonstrate any phenotypical differences from normals (154). It is possible, therefore, that paracrine and autocrine IGF-I production may be all that is required to sustain normal growth and development. In situ hybridisation studies have reported that IGF-I is located predominantly in stromal tissue, whilst IGF-II is expressed by both stromal cells and breast cancer cells (144;155;156). This suggests that IGF-I may be working in a paracrine fashion and similarly, IGF-II has been found to be over expressed in stromal cells surrounding breast cancers (157;158). A definite conclusion regarding its expression and localisation remains to be determined.

1.5.1 Circulating levels of IGF-I and IGFBP-3

IGF-I and IGFBP-3 are produced primarily in the liver and are thought to have a common, growth hormone regulatory mechanism. The production of IGF-I, IGFBP-3 and ALS is directly affected by GH. Previous studies have shown that reduced levels are seen in growth hormone-deficient states and more is seen in growth hormone-excess (159-161). Studies have shown, however, that IGF-I and ALS are produced by the hepatocyte and IGFBP-3 are produced in the non-parenchymal Kuppfer cells (162). It has been suggested, therefore, that the main site of GH regulation of the ternary IGF-binding complex is at the level of ALS and IGF-I synthesis and so changes in IGFBP-3 levels may be secondary to changes in IGF-I regulation or clearance.

Serum IGFBP-3 levels rise throughout early childhood, peaking in puberty, then gradually decline with age. They are generally higher in girls and are also positively correlated with height and BMI (Body Mass Index). Serum IGF-I levels show a similar increase from childhood to puberty but much larger than that seen with IGFBP-3. Levels then gradually decline with age and no difference is seen according to sex or

BMI (163;164). Significant differences in IGFBP-3 levels exist between early and late puberty and so there is a large reference range for interpretation of population IGFBP-3 and IGF-I levels. The sharper increase in serum IGF-I seen during puberty means that the molar IGF-I: IGFBP-3 ratio is increased. This suggests a possible differential regulation of IGF-I and IGFBP-3 in puberty and so the increasing IGF-I: IGFBP-3 ratio may reflect biologically active, free IGF-I (163).

Previous studies have demonstrated that there is a positive correlation between serum IGF-I and IGFBP-3 levels despite the paradox that IGF-I is positively correlated with cancer risk and IGFBP-3 is negatively correlated. This suggests that there may be a biological significance of individuals within the broad normal ranges between acromegaly (hypersecretion of growth hormone, IGF-I and IGFBP-3) and growth hormone deficiency. In contrast, other studies have shown that advanced cancers are associated with low levels of IGF-I which is thought to be as a result of cachexia and malnourishment. IGFBP-3 protease activity has also been shown to increase in advanced cancer states (65;165). The increase in cancer risk seen in individuals with high IGF-I/IGFBP-3 ratios may be due to differences in cell cycle kinetics and hence may impact on many extra divisions in epithelial cell populations in the long term (166). The need for further prospective studies in this area of research is therefore clear.

Anti-oestrogen Tamoxifen has been shown in several studies to reduce serum IGF-I (66;167-170). One study, however, showed that levels were unchanged (139) and another showed an increase in IGF-I with Tamoxifen treatment (171). Levels of IGFBP-3 in response to Tamoxifen, however, seem to be contradicting. Three studies report unchanged levels (165;167;171)whilst others have found increased levels (139;172). The effects of Tamoxifen are thought to be mediated by several pathways; inhibition of protein kinase C (173), stimulation of TGF-β (174)as well as anti-angiogenic effects (175;176). A recent study has

demonstrated that Tamoxifen reduced serum IGF-I levels and risk of recurrence (177), therefore, it may be possible that its effects on the GH-IGF axis are partly responsible for its beneficial effects in the treatment of breast cancer.

1.6 Modulation by other growth regulators

Recently it has been shown that the IGF-independent growth inhibitory effects of IGFBP-3 may involve interaction with other growth factor systems. For example, transforming growth factor-β (TGF-β) has been shown to have marked anti-proliferative effects on breast cancer cells which is also associated with an increased expression of IGFBP-3 mRNA and protein (178). Other studies have also shown that TGF-β induces expression of IGFBP-3 and that its growth inhibitory effects may be inhibited by antisense oligoncleotides to IGFBP-3 (179;180). A positive correlation has also been reported between IGFBP-3 and TGF-β1 in breast cyst fluid in women who also have a reduced risk of developing breast cancer (181). One study has proposed that the TGF-βV receptor may be a putative IGFBP-3 binding site (45).

Many studies looking at breast cancer cell lines have shown correlations between IGFBP-3 mRNA, protein expression and the growth inhibitory effects of TGFβ, anti-oestrogens (182), retinoic acid (183) and vitamin D analogs (184;185). Other studies have shown that other growth modulators such as anti-oestrogens, vitamin D and TNFα are able to induce production of TGF-β in breast cancers (186-188). It is possible, therefore, that TGF-β exerts its anti-proliferative effects on breast cancer cells via IGFBP-3.

1.7 Nuclear actions of IGFBP-3

IGFBP-3 has been localised to the nucleus of certain cells and contains a nuclear localisation sequence which may facilitate nuclear transport. (54;189;190). The nuclear role of IGFBP-3 is unclear, however, the cellular effects involve modulation of gene transcription. The retinoid X receptor (RXR) plays a key role in regulating gene transcription mediated by the following factors; thyroid receptors, peroximase proliferators activating receptors and the vitamin D receptor (191-194). The RXR can form a heterodimer with these nuclear transcription factors, for example the vitamin D receptor (VDR), which then bind to specific DNA sequences (vitamin D response elements, VDREs) which are located within the promoter region of responding genes (195).

Retinoic acid has been shown to inhibit the growth of breast cancer cells as well as up regulate IGFBP-3, 4 and 6 mRNA (196). It has also been recently reported that retinoid X-receptor may be a binding protein for IGFBP-3 and that it forms a complex with IGFBP-3 which is then taken into the nucleus after treatment with retinoic acid (23). In this study it was observed that the pro-apoptotic effects of IGFBP-3 requires an intact RXR signalling pathway as RXR knockout cells were unresponsive to IGFBP-3 induced apoptosis. This implies that the IGF-independent actions of IGFBP-3 mat be mediated through direct effects on gene expression.

There have also been many studies investigating the effects of vitamin D analogues on breast cancer cells. *In vitro* studies have shown that they inhibit proliferation and *in vivo* studies have shown that they cause regression of breast tumours (197-199). One study reported a link between the anti-proliferative effects of vitamin D with an increase in IGFBP-3 protein (184). Vitamin D has also been previously shown to

increase p53 protein expression, another possible mechanism by which it mediates its anti-proliferative effects (43).

In vitro studies have shown that anti-oestrogens have anti-proliferative effects on MCF-7 breast cancer cells with an increase in IGFBP-3 and 5 mRNA and protein levels (182;200). It appears, therefore that retinoic acid and vitaman D may exert their antiproliferative effects on breast cancer cells via up regulation of IGFBP-3.

The main ligand for the vitamin D receptor (VDR) is 1,25(OH)2D, which is converted from its precursor 25(OH)D by 25-hydroxyvitamin D-1-α hydroxylase (1αOHase). Binding of 1,25(OH)2D to the VDR results in its translocation to the nucleus where it acts as a transcription factor. In addition to this, enzyme 24 hydroxylase (24Ohase) is responsible for breaking down active vitamin D. Extra-renal sites of 1αOHase expression have been described but its in *vivo* expression in the human breast has not been investigated.

1.8 Aims of study

There is increasing evidence that IGFBP-3 is expressed in breast tissue and may influence the development of breast cancers in women. Epidemiological studies have shown that IGFBP-3 levels may have a role in breast cancer risk and prevention, however, further work is needed to elucidate its expression in breast tissue and also its potential IGF-independent, growth inhibitory effects. The conflicting evidence obtained from previous work suggests the need to clarify the expression and effects of IGFBP-3 in breast tissue.

The aims of this study were;

1. To determine and compare the expression of IGFBP-3 mRNA in the following groups;

i. Paired samples of normal and malignant breast tissue.

ii. Normal breast tissue from women undergoing surgery for benign disease such as fibroadenoma/hamatoma.

iii. Paired normal and malignant tissue from women with recurrent breast cancer.

2. To confirm mRNA expression at a protein level using immunohistochemistry.

3. To correlate the local IGFBP-3 expression with the expression of;

i.Other members of the growth hormone/IGF-I axis (IGF-I and IGF-IR)
ii.The vitamin D axis (vitamin D receptor, 1-α hydroxylase and 24-hydroxylase).
iii.Other genes associated in breast tumourigenesis: COX-2 (cyclo-oxygenase 2), VEGF (vascular endothelial growth factor).

4. To determine whether such expression correlates with the clinical behaviour of breast cancer: including clinico-pathological features (tumour size, grade, histological type, lymph node status, presence of vascular invasion, presence of ductal carcinoma in situ (DCIS) and hormonal status) and survival (follow-up of 20 months by end of study).

5. To investigate the serum IGF-I and IGFBP-3 levels in women with breast cancer and determine their IGF-I/BP-3 ratios.

6. To determine the physiologically relevant IGF-I independent effects of IGFBP-3 on apoptosis using an *in vitro* model of primary breast tissue culture.

2. Methods

2.1 Materials

2.1.1 Major

Tissue Homogeniser Ultraturrax T8 – GMBH & Co, Germany.

Rneasy RNA extraction kit, Qiagen Ltd, Crawley, West Sussex,UK.

RNA 6000 Nano Labchip kit (RNA quantification), Agilent Technologies UK Ltd, Cheshire,UK.

Ribogreen (RNA quantification), Molecular Probes, Amsterdam, The Netherlands.

Wallac Victor^2 1420 Multilablel Counter (plate reader), Perkin Elmer Life Sciences, Cambridge,UK.

Mx4000^{TM} RT-PCR multiplex quantitative PCR system, Stratagene, The Netherlands.

Brilliant^{TM} plus single step quantitative RT-PCR core reagent kit, Stratagene, The Netherlands.

Primers and probes designed by Primer Express software, ABI Perkin Elmer, Warrington,UK.

ABC kit, Vector Labarotories, USA.

NuaireTM DH Autoflow automatic CO2 Incubator, Triple Red Lab Technology Ltd, Oxfordshire, UK.

Balance, Sartorius 1800, Sartorius Ltd, Sussex, UK.

MTS proliferation assay, Cell Titer 96 AQ_{ueous} One Solution Assay, Promega, Southampton,UK.

LDH cytotoxicity detection kit, Roche Molecular Biochemicals, NJ, UK.

Caspase-3 protease assay, Apotarget, California, USA.

Immunofluorescence, TSA™, Perkin Elmer, USA.

2.1.2 Minor

RNA later, Ambion (Europe) Ltd, Huntingdon, UK.

Cryogenic 2ml vials, Corning Inc, NY,USA.

Scalpels blade no.10, Fisher Scientific UK, Loughborough, UK.

Pipette tips, Starlab, Helsinki, Finland.

24 well and 96 well microplates, Abgene UK.

Microcentrifuge tubes, Starlab, Helsinki, Finland.

Depex mounting medium, Gerr, Canada.

Ethanol (96%), VWR International Ltd, Lutterworth, UK.

Amphotericin (0.25µg/ml), Invitrogen Ltd, Paisley, UK.

Penicillin (10mg/ml), Invitrogen Ltd, Paisley, UK.

Streptomycin (10mg/ml), Invitrogen Ltd, Paisley, UK.

Polyclonal rabbit anti-IGFBP-3 antibody, Upstate, USA.

Dulbecco's Modified Eagle Medium, GibcoBRL, Paisley, UK.

Bovine serum albumin, Sigma Poole.

Human IGFBP-3, Gropep, Australia.

IGFBP-3 ELISA, Diagnostic Systems Labarotories, Texas, USA.

Doxorubicin, Sigma, Poole.

Human anti-caspase-3 antibody, R&D systems, USA.

2.2 Ethics

Local Ethical Committee Approval was obtained and consent forms were designed specifically for this study (appendix A). Full informed consent was obtained prior to collection of all samples and a copy of this form was filed in the patient's notes, in the Research Department, given to the patient and also given to the Pathology Department.

2.3 Tissue collection

2.3.1 Tumour specimens

Mastectomy (with breast cancer) and segmental mastectomy specimens were collected from 53 women at the time of surgery. A further 10 specimens were collected from women with recurrent breast cancer that had developed resistance to Tamoxifen. All specimens contained tumours that were clinically palpable (greater than 1cm in size). Specimens were placed on ice and transported swiftly to the Pathology department. Samples of breast tumour tissue and normal breast tissue, as far away from the tumour site as possible (approximately 8-10cm), were harvested and placed immediately into 'RNA later'(Ambion, UK). The samples were then snap frozen in liquid nitrogen and stored at -80°C.

2.3.2 Normal breast tissue specimens

These samples were collected from 17 woman undergoing breast surgery for benign disease or breast reduction surgery. All patients were age matched as far as possible to the patients within the tumour bank. Full informed consent was obtained for all specimens. At the time of surgery,

samples of normal breast tissue were harvested from the tissue removed at operation and placed into 'RNA later' and then snap frozen in liquid nitrogen. These samples were also stored at -80°C.

2.4 RNA extraction

Successful extraction of RNA requires disruption of the tissues, inhibition of the activity of RNases (enzymes that degrade RNA) and removal of any contaminating genomic DNA or proteins.

All tissue samples were stabilised in 'RNA later' prior to archival storage at -80°C. Use of this reagent allows immediate stabilisation of RNA thereby preventing any changes in gene-expression pattern that may occur with RNA degradation. All eppendorfs used had been previously autoclaved.

Tissue samples of approximately 20-30mg were subsequently excised after removal from 'RNA later'. Due to poor yield of RNA from adjacent normal breast tissue, sample size of these samples was increased to 50mg.

Qiagen 'Rneasy' kit was used to extract RNA. This technique allows isolation of RNA molecules longer than 200 nucleotides. This therefore provides mostly mRNA as most RNAs less than 200 nucleotides long are excluded. Lysis buffer RLT, containing guanidine thiocyanate and B-mercaptoethanol, was used. This buffer is effective in inactivating RNases to allow isolation of intact RNA. Tissue disruption was first achieved using a tissue homogeniser and then using mortar and pestle.

2.4.1 Tissue homogenization

500µl of buffer RLT was added to 30mg of frozen tumour or 700µl RLT to 50mg of frozen adjacent normal. This increase in buffer for the normal tissue is needed as normal breast tissue is difficult to lyse and more buffer is needed to complete homogenization and to prevent the subsequent minicolumns from clogging.

A rotor 'Ultra-turrax' homogenizer was used to disrupt the tissues for approximately 15-20 seconds at speed setting 4. Care was taken to avoid over-heating and foaming. The rotor homogenizer disrupts the tissues using turbulence and mechanical shearing. The sample was then snap frozen using liquid nitrogen. Some difficulties were experienced using the tissue homogeniser and normal breast tissue as the normal tissue had a high fibrous tissue and fat content. This lead to unsatisfactory disruption of the tissues prior to extraction. The different technique of mortar and pestle grinding was then employed for normal samples.

2.4.2 Mortar and pestle

Using this technique, each frozen sample was placed into a mortar containing liquid nitrogen. The sample was then ground to fine powder using a pestle and the powder then scraped into a chilled eppendorf using a round bottomed scalpel blade. 500µl or 700µl of buffer RLT was then added accordingly and the mixture shaken vigorously prior to snap freezing in liquid nitrogen.

For subsequent mRNA extraction, all samples were defrosted for 5 minutes in a water bath at 55 - 65°C. They were then placed in the centrifuge for 5 minutes at 13,000 rpm. The supernatant was carefully transferred into a new eppendorf, discarding any unhomogenized tissue.

An equal volume of 70% ethanol was then added to the supernatant. For example, 500µl to the tumour samples and 700µl to the normals. The samples were then mixed by vortexing. 700µl of the mix was then pipetted into the RNeasy mini column which was placed into a collection tube. The column and tube were then centrifuged at 9000 rpm for 1 minute. The mini column contains a silica-gel-based membrane which allows binding of total RNA whilst contaminants may be washed away. The protocol then states that the follow through may then be discarded. After several extractions with poor yield of RNA, it was discovered that it was beneficial to pipette the follow through back into the mini column and re-centrifuge for a further minute before discarding.

This process was then repeated for the remaining buffer/ethanol mix. 350µl of buffer RWI (ethanol containing) was then added to the mini column and the column left to stand for 15minutes with its lid open. After 15 minutes, the column was placed into the centrifuge at 9000 rpm for 1 minute. At this point a DNase step was performed. This step is optional but used for the purposes of this study as Taqman PCR analysis is sensitive to even small amounts of DNA.

2.4.3 DNase Step

A mixture of 10µl DNase I stock solution and 70µl buffer RDD was made up, for each sample. This was then mixed gently by inverting the tube. 80µl was then pipetted directly onto the membrane within the mini column and left, with lids open, for 15 minutes. After 15 minutes, a further 350µl of buffer RWI was pipetted into the mini column and the column then centrifuged at 8000 rpm for 1 minute. At this point the follow through was discarded and the mini column placed in a new collection tube.

500µl of RPE was then added to the mini column and centrifuged at 9000 rpm for 1 minute. The follow through was then discarded and a further 500µl RPE added, centrifuged at 9000rpm for 3 minutes and discarded.

The mini column was then centrifuged at 10 000 rpm for 3 minutes in order to dry out the membrane and then transferred into a new collection tube.

80µl of RNase free water was then added directly onto the membrane and left to stand for 2 minutes. The mini column was then centrifuged at 10 000 rpm for 3 minutes. A further 65µl of RNase free water was then added and the mixture left to stand for 5 minutes. The column was then centrifuged at 13 000 rpm for 4 minutes. 145µl of follow through was then collected into two new eppendorfs; 10µl in one eppendorf for RNA quantification and the remaining 135µl into another for archival storage at -80°C.

2.5 RNA analysis

There are many different methods of analysing and quantifying RNA. Usually, the quality is checked by gel electrophoresis whereby two distinct bands form; 28S and 18S. The ratio of 28S:18S should be 2:1 thereby confirming that the RNA has not undergone significant digestion by RNases. If the RNA has degraded, the ratio will be reversed. With this method of quality control, it is the ribosomal (rRNA) and not the messenger RNA that is checked as it is the rRNA which produces the strong bands. Messenger RNA only produces a faint smear on a gel.

Initially, the Ribogreen RNA quantification kit was used to quantify the RNA. It is a fluorescent nucleic acid stain that is able to accurately measure RNA up to as little as 5 ng/ml RNA.Those samples mostly included normal breast tissue which, due to its high fat content, tended to produce low yields of mRNA. Breast tumour tissue, however, produced much higher quantities of mRNA and so for these samples, the Agilent bioanalyzer was used.

2.5.1 Ribogreen RNA Quantification Kit

The Ribogreen method of RNA quantification is a sensitive fluorescent nucleic acid stain that may be used in conjunction with a fluorescence microplate reader. The Ribogreen reagent is an unsymmetrical cyanine dye which demonstrates a >1000 fold increase in fluorescence when bound to RNA. Free nucleotides and contaminating proteins do not interfere with the measurement. It is essential that all samples have had DNA removed as it also binds to the Ribogreen reagent.

Samples to be quantified were removed from -80°C storage and kept on ice. Clean disposable gloves were used throughout to minimise contamination from RNases. TE assay buffer was provided at 20x strength required. It comprised 10mM Tris-HCL and 1mM EDTA at pH 7.5 and was diluted to 1/20 using nuclease-free water (2ml TE was added to 38ml water). Nuclease-free water was prepared by treatment of distilled, de-ionized water with 0.1% diethyl pyrocarbonate (DEPC) followed by incubation for 2-3 hours at 37°C and then autoclaved for 15 minutes. This sterilizes the water and eliminates the DEPC.

2.5.1.1 Tumours

400µl of TE was pipetted into an autoclaved eppendorf. One eppendorf for each sample to be analysed. 2µl of tumour RNA was then pipetted into the TE buffer.

2.5.1.2 Normal tissue

300µl of TE was used for each sample of normal tissue. 5µl of normal RNA was then pipetted into the TE buffer.

The Ribogreen reagent was then made into a 1 in 200 solution with TE buffer and protected from light. This involved pippetting 50µl of Ribogreen into 9.95ml TE buffer and mixing by pipetting. 200µl of the Ribogreen solution was then added to each of the above tumour or normal tissue samples. 100µl of this mix was then pipetted into three consecutive wells within a 96 well plate and incubated for 2-5minutes.

2.5.1.3 Standard curve

A standard curve was then prepared to display the linear correlation between RNA concentration and fluorescence. Ribosomal RNA was provided at 100µg/ml and diluted to 2µg/ml using 10µl of standard RNA in 490µl TE buffer.

The following solutions were then prepared:

Vol of 2µg/ml RNA(µl)	Vol of TE (µl)	1:200 Ribogreen agent	Concentration of RNA (ng/ml)
160	40	200	800
120	80	200	600
100	100	200	500
80	120	200	400
60	140	200	300
40	160	200	200
20	180	200	100
10	190	200	50
0	200	200	0(blank)

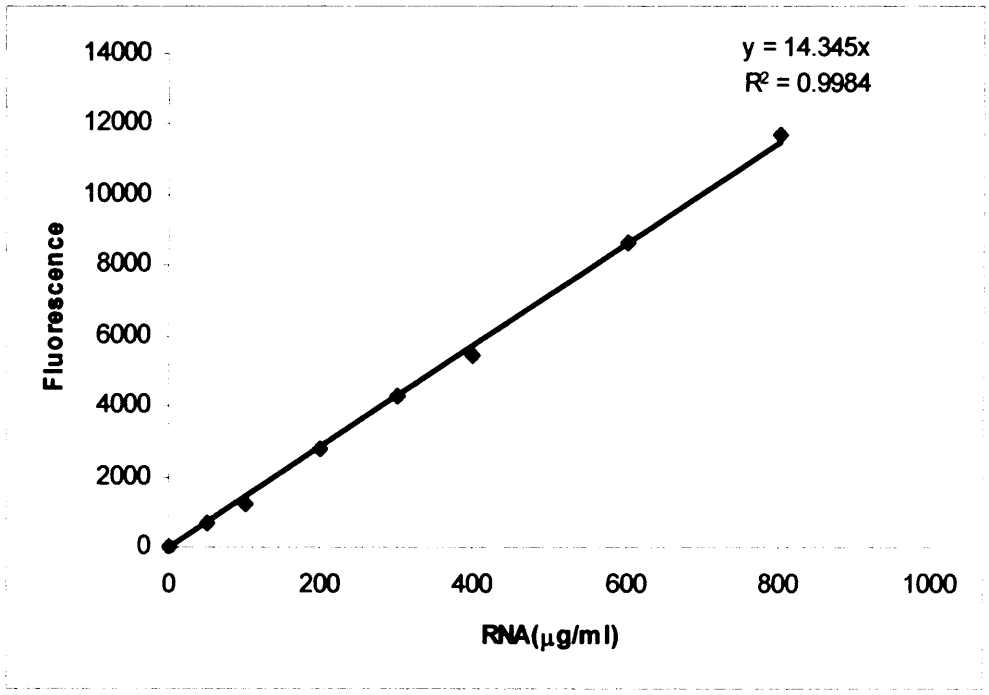
At each concentration, 100µl of solution was pipetted into 3 consecutive wells within the above mentioned 96 well plate and incubated for 2-5 minutes.

The 96 well plate containing the sample RNA and the standard curve was then placed in a fluorescence microplate reader and measured at 450-520 nm. The instrument's gain was set so that the sample containing the highest RNA concentration yielded a fluorescence intensity near to the maximum. This ensured that all sample readings remained in the detection range.

First, the average of all three fluorescence values was calculated for all samples. Then the fluorescence value of the blank reagent was subtracted from each of these values. For the standard curve graph, this

value was then plotted as a scatter graph against the RNA concentrations (Fig 2). From the plots on the graph a trend line was then added and from this the y-intercept value was calculated.

Figure 2. Representative standard curve of sample RNA from a breast cancer (each value is the mean of three fluorescence values) quantified using the Ribogreen assay. Y intercept value is 14.3 and R value >0.99.



2.5.1.4 Calculation of RNA from standard curve

The mean of three fluorescence values were calculated per sample. The fluorescence value of the blank reagent was then subtracted from this value. This value was then used to calculate the concentration of RNA according to the dilution factor used in making up the RNA/TE and ribogreen solutions;

2.5.1.4.1 Tumour

[Fluorescence/y–intercept] x [400/2] x [400/200] = concentration of RNA ng/ml.

2.5.1.4.2 Normal

[Fluorescence/y-intercept] x [300/5] x [400/200] = concentration of RNA ng/ml.

2.5.2. Agilent Bioanalyzer

The RNA 6000 Nano LabChip Kit simultaneously measures quantity and quality of messenger or total RNA (Fig 3). It comprises a lab chip and a bioanalyzer attached to a PC. Each sample is injected into a well and then moves sequentially from its well to a central separation microchannel, where it is separated electrophoretically. As the fragments move down the central separation channel, they separate by size and are detected by fluorescence and recorded by the bioanalyzer machine.

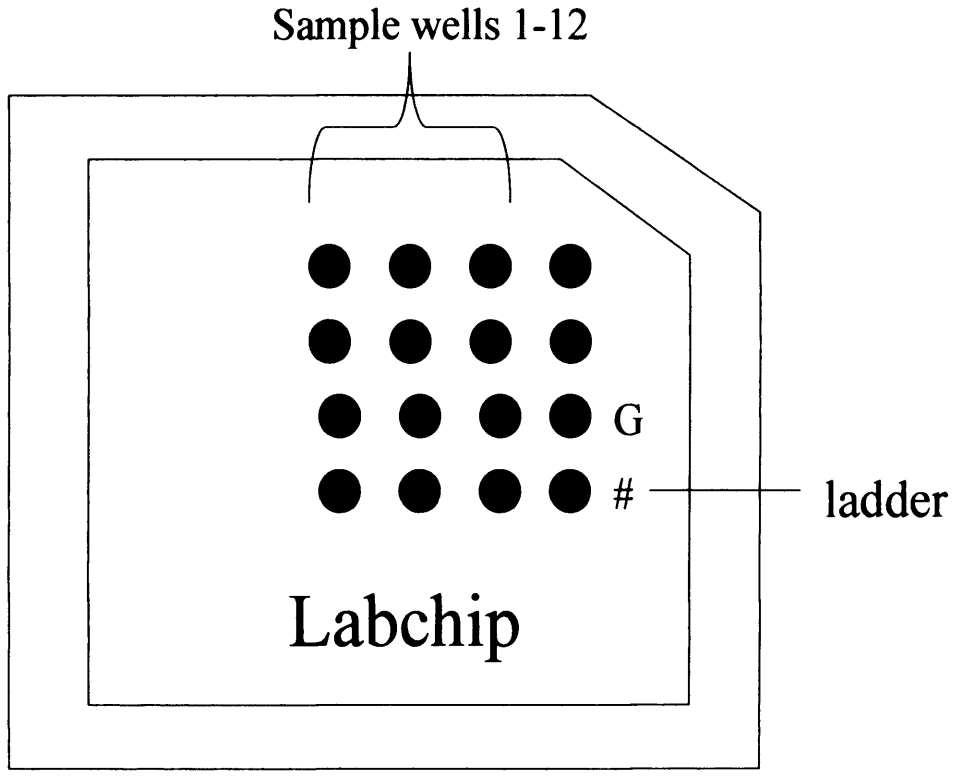
Firstly, samples were taken from -80°C storage and placed on ice. The protocol recommends that all RNA samples should be heat denatured at 70°C for 2 min, however, due to poor yield of RNA this practice was not continued.

The Bioanalyzers electrodes were cleaned prior to each chip. This was performed using 350µl RNase Zap followed by 350µl RNase-free water pipetted into an electrode cleaner and then into the bioanalyzer.

The gel was prepared by pipetting 550µl of RNA 6000 Nano gel matrix into the top receptacle of a spin filter. The spin filter was then centrifuged at 4000 rpm for 10 minutes. 65µl of filtered gel was then pipetted into a new, autoclaved eppendorf. 1µl of 6000 Nano dye concentrate was then added to the 65µl of filtered gel and the eppendorf vortexed. Proper mixing was inspected visually. The eppendorf was then centrifuged for 10 minutes at 14 000 rpm.

The chip priming station base plate was placed in position 'C' and an RNA Nanochip placed within it. 9µl of the gel dye mix was pipetted into the bottom of the well marked 'G.' Care was taken to avoid bubbles. The plunger was then advanced to the 1ml level, the chip priming station was closed and the plunger held in place by the syringe clip. This remained in position for 30 seconds. After 30 seconds, the plunger was released and the plunger allowed to resume its usual position. After 5 seconds, the plunger was slowly pulled back if it hadn't already resumed its normal position. The priming station was then opened and 9µl of gel dye mix was pipetted into the two wells above the one marked 'G.'

Figure 3. RNA Nano Labchip in chip priming station. Each chip analyzes 12 samples simultaneously. G represents the well containing the gel-dye mix and # represents the well containing the ladder.



5µl of RNA 6000 Nano Marker was pipetted into the well marker 'ladder' and then into each of the 12 wells consecutively.

2.5.2.1 Loading the ladder and samples

3µl of RNA 6000 ladder was pipetted into an autoclaved eppendorf and denatured at 70°C for 2 minutes. 1µl was then pipetted into the well marked 'ladder.'

1µl of each of the samples to be analysed was then pipetted into each of the sample wells labelled 1 to 12. The chip was then placed into the vortex mixer and vortexed for 1 minute at set point (2400 rpm).

After vortexing, the chip was then placed into the Agilent 2100 Bioanalyzer. The lid was closed. The software instructions on the accompanying PC were then followed; the correct assay selected, the sample wells labelled and the 'start' button clicked to commence the assay. All RNA concentrations were then collected from the PC at the end of the assay.

2.7. Real time reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR is a sensitive, *in vitro* method used to detect messenger RNA (mRNA). It is used to measure the abundance of specific RNA or DNA sequences in clinical samples and may also be used to screen for mutations and single nucleotide polymorphisms. The Taqman assay has been used to quantify transcription from individual cells (201) and paraffin embedded tissue (202). It has wide ranging clinical applications and has been used to screen serum for genetic diseases (203), detect the presence of viral and bacterial pathogens (204-207) and has been useful in the detection of erb-B2 transcription in breast cancer (208).

The technique relies on having unique, known sequences of DNA either side of the segment to be amplified. Short DNA oligonucleotides complementary to the flanking sequences are required and are known as primers. DNA may be heated in order to denature the double helix and become single strands. Upon cooling, double stranded DNA reforms. If an excess of oligonucleotide primers complementary to the sequences flanking the segment of interest are added, they will preferentially anneal to the segment and with the addition of a DNA polymerase enzyme, a second strand complementary to this segment will be produced. The DNA polymerase of the thermophile algae Thermus aquaticus (Taq.) is stable at the temperatures required to cause dissociation of double stranded DNA (209). Use of Taq polymerase allows a rapidly repeating cycle of denaturation, primer annealing and sequence extension to occur and is known as a polymerase chain reaction (PCR). It is a process that was first reported in 1985 (210) and has since been modified to incorporate Taq polymerase and an automated thermocycler (211).

The PCR reaction amplifies DNA. mRNA may be reverse transcribed in order to form complementary DNA (cDNA) using a reverse transcriptase (RT) enzyme. RT enzymes were originally derived from retroviruses and are now manufactured from cloned viral genes. Using this technique, it possible to rapidly detect very small amounts of mRNA.

The first step in the assay is the enzymatic conversion of RNA to a single-stranded cDNA template. This is followed by exponential amplification in a PCR reaction (Fig 4). Dedicated RNA and DNA dependent DNA polymerases may be used in single or separate reactions. This allows for the customisation of individual RT-PCR assays. Seperation of the RT and PCR steps allows a stable library of cDNA to be generated which may be stored for long periods, alternatively a single polymerase which acts as both an RNA and DNA-dependent DNA polymerase may be used in a single reaction thereby minimising error and risk of contamination.

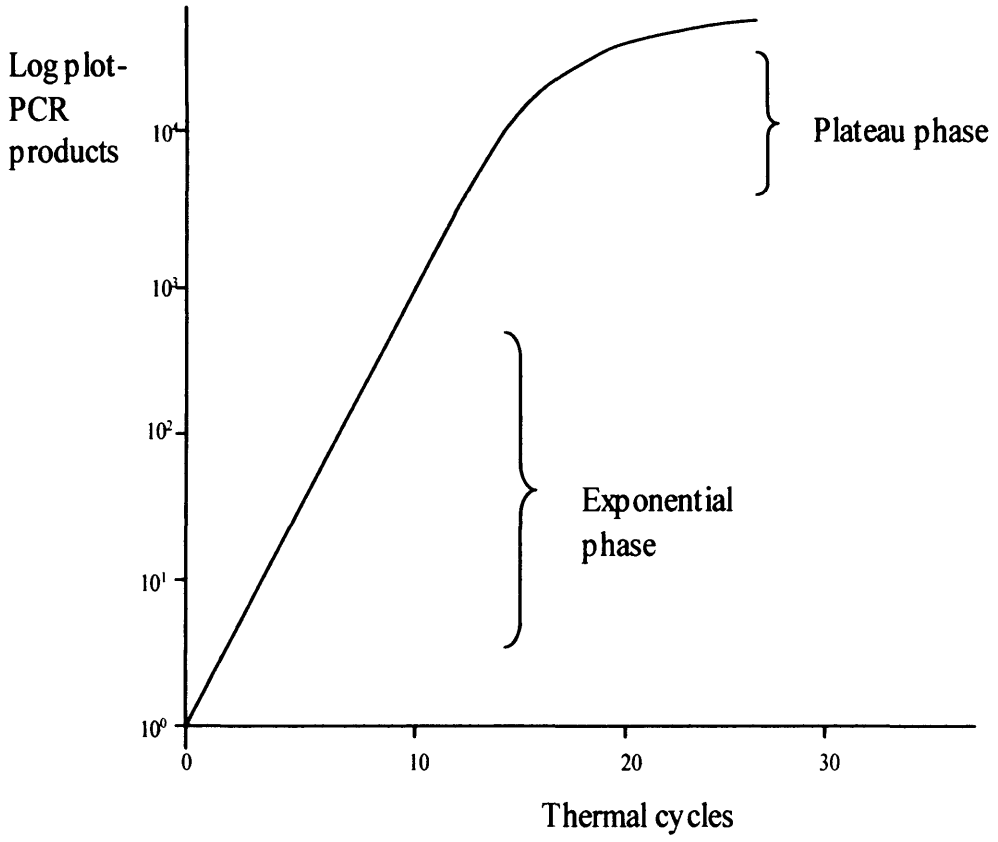
2.7.1 Reverse transcription

Two commonly used reverse transcriptases (RTs) are avian myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). RNA transcripts may have significant secondary structure that affects the ability of the RNA-dependent DNA polymerase (RT) to generate transcripts (212) and may also affect RT-PCR quantification. AMV-RT is the more robust RT, retains significant polymerisation up to 55°C and may also eliminate any problems associated with RNA secondary structure. MMLV-RT, however, has significantly less RNAse H activity which can interfere with the synthesis of long amplicons (213;214).

The RT step may be primed by specific primers, random hexamers or oligo-dT primers. Use of random hexamers and oligo-dT primers can

maximise the number of mRNA molecules that may be analysed from a very small sample of RNA. mRNA specific primers were used for the purposes of this research, however, since they decrease background priming and use of random hexamers can overestimate mRNA copy numbers up to 19-fold (215).

Figure 4. PCR reaction. The graph demonstrates product accumulation during the exponential and plateau phases of the reaction (log scale).



2.7.2 The Polymerase Chain Reaction

A variety of thermostable DNA-dependent polymerases are available and differ in their processivity, thermal stability, fidelity and ability to read modified triphosphates such as deoxyuridine and deoxyinosine in the template strand (216;217).

The most commonly used enzyme, Taq DNA polymerase was used for the purpose of this research. It has an oligonucleotide which is labelled at one end (5′) with a fluorescent group and a quencher at its 3′ end (Fig 5). It has a 5'-3' nuclease activity but lacks a 3'-5' proofreading exonuclease activity.

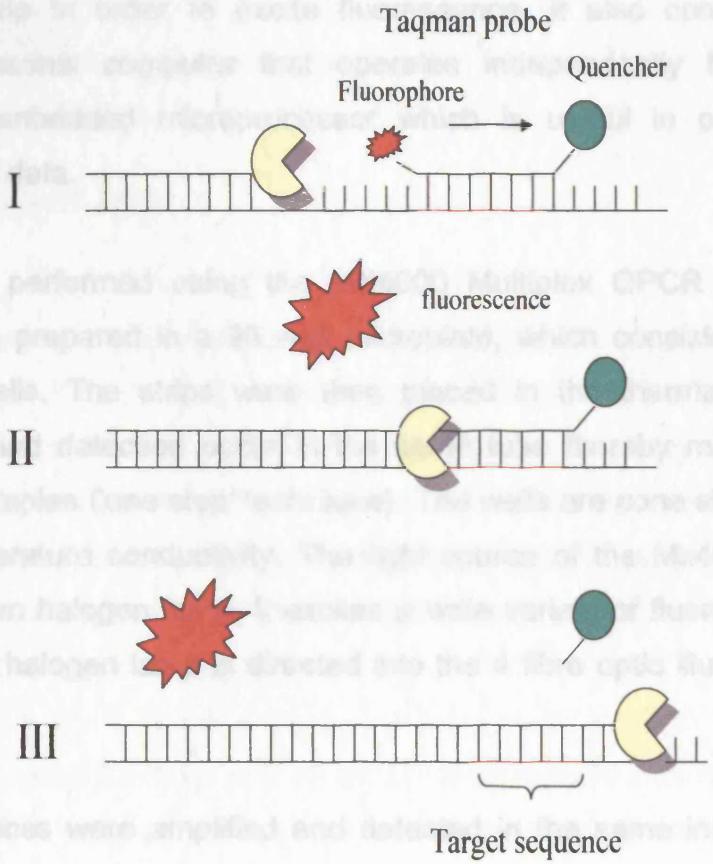
The labelled oligonucleotide and primers were added to the PCR assay. When the fluorescent and quenching groups are close together, the emissions from the reporter dye are absorbed by the quencher dye and so the fluorescent emission is low. As the DNA is amplified, an increasing amount of oligonucleotide probe hybridises to the denatured DNA. During the extension phase of the PCR cycle, the 5′- 3′ exonuclease activity of polymerase cleaves the fluorophore from the probe. As the fluorophore moves away from the quencher, there is an increase in fluorescence. This is measured in proportion to the amount of DNA synthesized during the PCR. The Stratagene Mx4000, as used in this study, contains photomultiplier tubes in order to detect the fluorescence.

2.7.3 One-enzyme RT-PCR

The one enzyme system is useful where target RNA contains extensive secondary structure. It utilises *Thermus thermophilus* (T_{th}) polymerase which is a DNA polymerase with intrinsic RT but no Rnase H activity (218). This technique is particularly useful in reducing

the amount of hands-on time in the experimental set up and therefore minimises the potential contamination of reaction mixtures. A study has also shown that RT-PCR reactions using Tth polymerase may be more robust and resistant to inhibitors (219). The drawbacks of this technique are that it is not as sensitive as the two-enzyme technique (220;221),the presence of Mn^{2+} ions reduces the fidelity of nucleotide incorporation (222), Tth polymerase lacks a 3'-5' exonuclease proofreading activity and first strand synthesis must be initiated from specific rather than random primers.

Figure 5. Polymerase chain reaction using Taqman technology. I: oligo labelled with fluorescent group, II: polymerase cleaves the fluorophore from probe, III: probe fluoresces as DNA polymerase seperates fluorophore from quencher.



2.7.4 Instrument

The Mx4000^{TM} real-time multiplex quantitative PCR system was used for this study as it accurately quantifies mRNA. It has a high specification and is easy to use. It is a fluorescence based kinetic technique that uses a halogen lamp in order to excite fluorescence. It also contains an integrated personal computer that operates independently from the instrument's embedded microprocessor which is useful in protecting against loss of data.

All PCR was performed using the MX4000 Multiplex QPCR System. Samples were prepared in a 96 well microplate, which consisted of 12 strips of 8 wells. The strips were then placed in the thermal cycler. Amplification and detection occur in the same tube thereby minimising handling of samples ('one step' technique). The wells are cone shaped to improve temperature conductivity. The light source of the Mx4000 is a quartz, tungsten halogen lamp. It excites a wide variety of fluorophores. Light from the halogen lamp is directed into the 4 fibre optic illumination paths.

Target sequences were amplified and detected in the same instrument and the rate of accumulation of amplified DNA was measured by the machine over the course of the PCR assay. The greater the concentration of target sequences, the fewer the number of cycles required to achieve a yield of amplified product. The concentration of target sequences may be expressed as the fractional cycle number (C_t) required to achieve a threshold of amplification. C_t may then be plotted against the log of the copy number to give a standard curve. This graph may then be used to quantify target sequences in unknown samples.

Fluorescence emission is collected from each of the QPCR samples as a result of light from the fibre optic illumination paths. The emission is

collected by fibre optics, filtered spectrally and detected by 4 photomultiplier tubes (PMT). Each PMT has an individual gain that automatically adjusts to the specific signal of a given dye.

The emission data is collected every few seconds and the data analysed by a software package on a nearby connected computer.

2.7.5 Optimisation

2.7.5.1 One step RT-PCR

The Brilliant™ plus single-step quantitative RT-PCR core reagent kit was used for all RT-PCR assays. The single-step technique is useful for performing cDNA synthesis and PCR amplification in one tube and using one buffer. This permits the RT reaction to be carried out at increased temperatures using primers and significantly higher melting temperatures, while reducing both hands on time and the likelihood of introducing contaminants into reaction mixtures.

The kit also includes Stratascript™ reverse transcriptase which is a novel Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) without any RNase H activity. This mutant of MMLV-RT is able to produce larger yields of full length cDNA transcripts than wild type MMLV-RT which has substantial RNase H activity. RNase H has a highly undesirable degradative activity which can affect the reverse transcriptase function.

Surestart™ Taq DNA polymerase is used as part of the single-step technique. It is a modification of Taq2000™ DNA polymerase with hot start capability. Surestart™ Taq is able to reduce background and increase amplification of desired PCR products.

The main drawback of this technique is that it may not be as sensitive as the two-enzyme technique (220;221).

2.7.5.2 Primers and probes

The probes used in this study included IGFBP-3, IGF-I, VEGF 189 (isoform associated with liver metastases), COX-2, VDR, 1-α-OHase and 24 OHase. They were newly designed using Primer Express software and are shown in Table 1.

Taqman probes are linear with the fluorophore at the 5′ end and the quencher either at the 3′ end or internal. If the probe is intact, no fluorescence is emitted from the fluorophore. During the annealing phase, the primers and probe hybridise with the target. The DNA polymerase then displaces the probe and the 5′ nuclease activity of the DNA polymerase separates the fluorophore from the quencher. During each PCR cycle, fluorescence may then be detected as it accumulates. The probes should have a melting temperature of 7-10°C higher than that of the primers. The ideal concentration should be determined empirically, ideally being the lowest concentration which results in the lowest C_t with an adequate fluorescence.

Probe concentration is usually in the 100nM range and they must compete for binding to their target with the amplification primers which are present in greater concentrations. As amplification primers are extended as soon as they bind to their targets, the hybridisation target sequence is rapidly masked with newly synthesized DNA. Therefore, the melting temperature (T_m) of the probe must be significantly greater (approximately 10°C) than that of primers, to ensure that they hybridise before the primers. Most probes used here were approximately 30 bases in length with a G/C content of approximately 50%. This ensures good specificity and a T_m high enough to be useful under PCR conditions.

Primers should bind to separate exons to avoid false positive results arising from amplification of contaminating genomic DNA. RNAse-free DNAse was used in the purification of RNA preparations as the intron/extron boundaries were unknown. Primer selection is based on estimated melting temperatures (T_m), the desire for small amplicon size and location of the probe. They must be designed to exact specifications and the Taqman technology provides its own primer and probe design programme known as Primer Express.

The optimal concentration of upstream and downstream primers is also the lowest concentration which results in the lowest C_t with an adequate fluorescence. When used with Taqman probe, the primer concentration may be varied between 50 to 300 nM and should be determined empirically. Too great a primer concentration may result in mispriming and accumulation of non-specific product. Too low a primer concentration is less problematic for real-time assays as target copy numbers will have been calculated well before the primer supply is used up.

The optimal length for single stranded primers is approximately 15-20 bases, the G/C content should be between 20-70% and their T_m should not differ by more than 1-2°C. The minimum T_m should be 58°C and the maximum should be 60°C. PCR primers were designed with similar annealing temperatures for all targets to be amplified.

Table 1. Table of primer and probe sequences. Forward (F) and reverse (R) primers and Taqman probes (P) used in RT-PCR.

Target gene	Primer/ Taqman probe	Sequence (5' – 3')
IGFBP-3	F	-AGA ACA GAT ACC CAG AAC TTC TTC-
	R	–CGC CCT CCG ACT GCT G-
	P	TTT TAT TTC AAC AAG CCC ACA GGG TAT-GGC
IGF-I	F	-CTT CAG TTC GTG TGT GGA GAC AG-
	R	-CGC CCT CCG ACT GCT G-
	P	-TTT TAT TTC AAC AAG CCC ACA GGG-TAT GGC-
VEGF 189	F	-TGT GAA TGC AGA CCA AAG AAA GA-
	R	-CGT TTT TGC CCC TTT CCC-
	P	-AGA GCA AGA CAA GAA AAT CCC TGT-GGG C-
COX-2	F	-GAA TCA TTC ACC AGG CAA ATT G-
	R	-TTT CTG TAC TGC GGG TGG AAC-
	P	-TTC CTA CCA CCA GCA ACC CTG CCA-
VDR	F	-ATC TGC ATC GTC TCC CCA GAT-
	R	-AGC GGA TGT ACG TCT GCA GTG-
	P	-TGA TTG AGG CCA TCC AGG ACC GC-
1αOHase	F	-GCT ATT GGC GGG AGT GGA C-
	R	-GCC GGG AGA GCT CAT ACA GA-
	P	-CCC AAG AGA GCG TGT TGG ACA CCG-
24 OHase	F	-TCC TTT GGG TAA AGC ATA TTC ACC-
	R	-GCC TGT CTG AAA GAA TCT ATG AGG-
	P	-AAC TGT TGC CTT GTC AAG AGT CCG

2.7.5.3 Magnesium chloride

Magnesium chloride concentration requires strict control as it can affect the specificity of the PCR primers and probe hybridisation. The ideal concentration is that which results in the lowest Ct and the highest fluorescence at a given target concentration. The $MgCl_2$ concentration may be titrated from 2-6mM with increments of 0.5 mM; in this case, $MgCl_2$ concentration was 3mM.

In addition, $MgCl_2$ can increase the melting temperature (T_m) of double stranded DNA and forms soluble complexes with dNTPs which can produce the actual substrate that the polymerase recognises. High concentrations of dNTPs therefore interfere with polymerase activity and affect primer annealing by reducing free $MgCl_2$.

2.7.5.4 Internal reference dye

A passive reference dye was used to provide a stable baseline to which the samples were normalised. The dye provided was diluted to 1:200 and a fresh solution made up for each assay.

2.7.5.5 Threshold cycle (C_t)

With every PCR assay, fluorescence values are recorded during each cycle and represent the amount of product amplified to that point in the amplification reaction. The greater the RNA template at the start of the reaction, the fewer the number of cycles taken to reach the point in which the fluorescent signal is statistically higher than the background value (223). This point is referred to as the C_t and always occurs during the exponential phase of amplification.

The reporter signal is normalised to the fluorescence of an internal reference dye to allow for corrections in fluorescent fluctuations caused by changes in concentration or volume, and a Ct value is reported for each sample. This value may then be translated into a quantitative result by constructing a standard curve.

2.7 6 PCR Reaction and conditions

The Brilliant™ plus single-step quantitative RT-PCR core reagent kit was taken from storage at -80°C and defrosted on ice. The reference dye was diluted to a solution of 1:200 with DEPC water. Each experimental reaction was made up to a volume of 25µl per sample. Each reaction was performed in duplicate, therefore the final volume per sample was 50µl.

The following reagents were then pipetted, in order, into an autoclaved eppendorf (a master mix for all samples was made up at the beginning of an experiment to reduce pipetting error);

2.5µl of 10x core RT-PCR buffer
1.5µl of 50nM magnesium chloride
2µl of dNTP mix
1.2µl of IGFBP-3 primer
0.25µl of Stratascript reverse transcriptase
0.25µl of SureStart Taq DNA polymerase
0.38µl of diluted reference dye
0.4µl of IGFBP-3 probe
10.5µl of nuclease-free DEPC water (to make solution up to 25µl).

This solution was then placed in the centrifuge at 10,000rpm for 5 minutes. 46µl of the mix was then pipetted into each individual microcentrifuge tube and 4µl of experimental RNA was added. For each

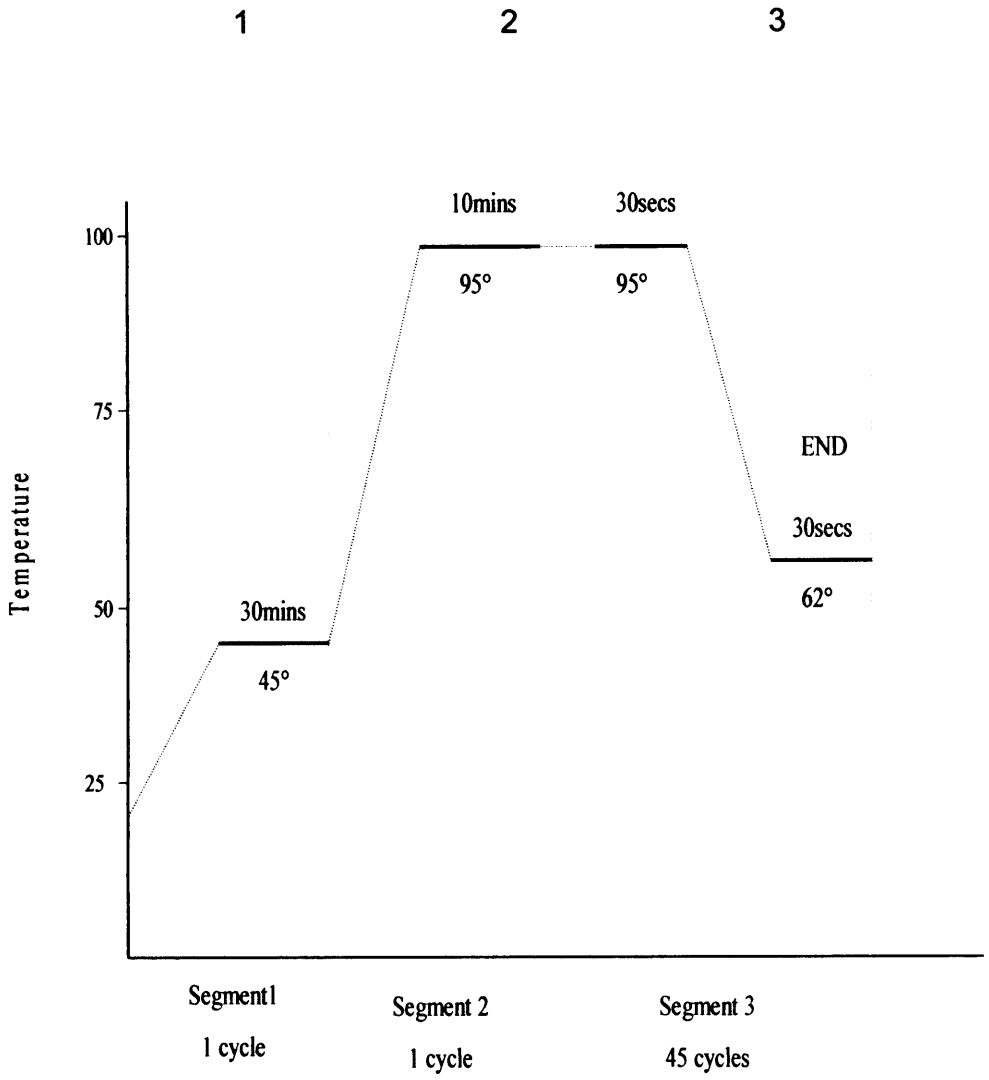
assay, 2 positive controls (liver RNA), 2 negative controls (water) and approximately 6 amplicons (serially diluted) were run in parallel.

Each one was then mixed by pipetting up and down and the pipette tip was discarded each time. The microcentrifuge tubes were then placed in the centrifuge for 5 minutes at 300 rpm. 25µl of each experimental reaction was then aliquotted into duplicate microcentrifuge tubes. The optic caps were then carefully and firmly positioned onto the microcentrifuge tubes. The tubes were then placed into the centrifuge for a further 5 minutes at 300 rpm.

Taking care not to disturb the samples, the microcentrifuge tubes were then transfered into the thermal plate. The reaction and thermal profile were then set up using the software package on the connecting PC. The thermal profile used for each PCR reaction is shown below (Fig 6).

The data was collected at each 'END' point. Using the software, it was possible to determine the C_t values for each sample (performed in duplicate).

Figure 6. Thermal reaction conditions for each PCR reaction. Stages 1 and 2 = RT, stage 3 = PCR (45 cycles).



2.7.7 Quantification

Quantification of mRNA may be performed by quantitative or semi-quantitative methods (224). Commonly, semi-quantitative methods are employed by sampling the RT-PCR reaction mixture and dot-blot analysis (225) which are not precise and laborious. Quantitative techniques may be competitive or non-competitive (226;227).

Non-competitive RT-PCR involves the co-amplification of the target with a second RNA molecule under reagent concentrations and conditions so that there is no competition between target and standard (228). In these circumstances, quantification may be unreliable as the efficiencies of the RT and PCR steps may vary. Competitive quantification involves spiking into the RNA samples before RT of known amounts of RT-PCR amplifiable competitors that should be synthetic RNA molecules. The internal standard shares the same primer recognition and internal sequences as the primary target and so competes for reagents. Both should be amplified with the same efficiency as they are virtually identical and their amplicons may be distinguished by the addition of a restriction enzyme site to the standard (229) or by varying its size (230). A series of PCR tubes containing the target are spiked with serial dilutions of known copy numbers (10^7, 10^6, 10^5 etc) of the internal standard. The higher the concentration of the internal standard, the more likely the primers will bind and amplify it rather than the target. Gel electrophoresis may therefore be performed and a comparison can be made of ethidium bromide stained standard and target amplicons to allow target quantification (231). There is still, however, approximately 10% error and this method may still not result in absolute quantification as differences in amplification efficiency between target and competitor will remain undetected.

2.7.7.1 Normalisation

It is well established that an appropriate standard is used within each PCR experimental assay. The accepted method is to amplify a cellular RNA at the same time as the target, which serves as an internal reference against which other RNA may be normalised (232). The ideal standard should be expressed at a constant level among different tissues, at all stages of development and should be unaffected by experimental treatment (233). The problems with this method is that errors easily occur in the amount of starting material between samples. There may also be differences in the amount of RNA obtained from different tissue types e.g normal and malignant tissue, and differences also exist when samples have been taken from different patients. Therefore, obtaining an ideal standard that accommodates these differences is an important part of the assay.

Three commonly used RNAs to normalise gene expression include the mRNAs specifying the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and ribosomal RNAs (rRNA). GAPDH is ubiquitously expressed by most tissues and its expression has been shown to be constant at different times throughout experimental manipulation (234;235). Evidence does suggest, however, that GAPDH concentrations vary greatly amongst individuals (236), during pregnancy (237), with developmental stage (238;239) and during the cell cycle (240). It has also been shown to be up regulated in tumours and this variation, therefore, suggests that its use as a normal control may be inappropriate.

β-actin is also moderately expressed in most cell types. It is traditionally used as an internal standard in PCR assays. Several studies, however, have shown that transcription levels may vary widely in breast epithelial cell culture studies (241). rRNAs constitute 85-90% of total cellular RNA are generated by a distinct polymerase (242) and their levels are less

likely to vary under conditions that affect the expression of mRNAs (243). rRNA transcription can, however, be affected by biological factors, drugs and may vary amongst individuals (241). It is also expressed at much higher levels than target mRNAs and cannot be used for normalisation when targets have been enriched for mRNA as it is lost during mRNA purification.

There are obviously inherent problems associated with normalising to an internal standard and with comparing copy numbers between samples obtained from different individuals or tissues. In this study, we normalised copy numbers relative to total RNA concentration. Little is known, however, about the total RNA content per cell of different tissues *in vivo* or how this may vary between individuals and between normal and malignant tissue. Bustin *et al* addressed some of these issues by obtaining blood from healthy patients and counting and purifying nucleated blood cells. RNA content was quantitated and little variation was observed in amount of total RNA per cell thereby confirming the validity of this technique in comparing results between individuals (244).

RNA concentration was determined accurately using the Agilent Bioanalyzer and also confirmed using Ribogreen quantitation as described earlier. Copy numbers of target genes were then recorded as copy number per µg total RNA. The drawbacks of this approach include the fact that total RNA may be increased in highly proliferating cells and so this may affect the accuracy of comparing absolute copy numbers between normal and malignant tissue (233). In order to compare our normalisation technique with the standard housekeeping gene method, GAPDH mRNA expression was measured on all samples according to the PCR protocol.

2.7.7.2 Generation of standard curves

Standard curves were constructed with each experimental reaction in order to determine the absolute quantification of mRNA transcription. This method allows the copy number per cell to be determined. A standard curve was constructed for each amplicon thereby ensuring accurate reverse transcription and PCR amplification profiles(233). The amplicons were diluted serially as follows; 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2} and 10^{1} using TE buffer. These samples were then run alongside each RT-PCR assay and the C_t values obtained were used to construct an absolute standard curve . A linear graph was obtained from three independent serial dilutions of amplicon and assaying each dilution in duplicate, together with positive and negative controls. This allows the copy number per cell to be determined from absorbance. The dilutions were made over the range of copy numbers expected from the target mRNA expected in the experimental samples in order to maximise accuracy.

The construction of a standard curve for each target amplicon allows a quantitative method for detecting differences in gene expression. Differences may be detected in amplification efficiency and sensitivity.

2.8 Immunohistochemistry

2.8.1 Introduction to immunohistochemistry

Immunohistochemistry allows the identification and localisation of proteins on tissue sections using antibodies linked to enzymes that produce colour changes in indicator dyes. This involves the binding of a primary antibody to the antigen and secondly, the detection and visualisation of the bound antibody by one of a variety of enzyme based chromogenic systems. The type of chromogenic substrate depends on the type of enzyme used e.g aminoethyl carbazole (AEC) and diaminobenzidine (DAB) may be used with horseradish peroxidase. DAB substrate has high sensitivity and produces an insoluble, permanent brown precipitate which has a high contrast in photographs. Amplification of the staining may be achieved by using antibodies and enzymes which combine through binding proteins such as the avidin biotin system. This system essentially involves three steps; primary antibody, biotin-labeled secondary antibody and avidin-biotin-peroxidase complex. A lattice-like complex is therefore produced which is attracted to the sites of biotin-labeled antibody to give sensitive staining (245).

2.8.2 Tissues and its preparation

2.8.2.1 Tumour bank

Immunohistochemistry against IGFBP-3 (Table 2) was performed on 3µm sections of paraffin embedded blocks of 24 paired samples of breast tumour and adjacent normal tissue which had previously undergone RNA

analysis. The avidin biotin complex (ABC) immunohistochemistry method was used (246).

Table 2. Optimal dilution for primary BP-3 antibody used for immunohistochemistry.

		Human tissue	
Antibody against	Type	F	P
IGFBP-3	Pab	-	1:200

F = frozen tissue

P = paraffin embedded tissue

- = not tested

Pab = Polyclonal rabbit antibody (purified IgG from rabbit antiserum, does not recognise IGFBPs 1,2,4,5 or 6) (247;248).

2.8.3 Protocol for DAB staining

Tissues were prepared using standard fixation and paraffin embedding techniques courtesy of the Pathology Department, St Bartholomew's Hospital. Paraffin sections were then dewaxed and rehydrated according to the following standard procedures. For each specimen, one slide was stained as per protocol and one was used as a negative control and so was treated with just horse serum and no antibody. Sections of paraffin embedded human liver were used as a positive control.

2.8.3.1 Standard dewaxing procedure

The slides were placed into a metal rack. The rack was then placed in a glass jar containing xylene for 5min. The rack was then placed into another container of xylene for a further 5 min. The samples were then rinsed in 100% absolute alcohol for 3 min, then in separate containers 100% alcohol 3 min, 90% alcohol for 3 min, 70%, 3 min. The slides were not allowed to dry between any of the steps.

100ml of 10x citrate buffer was added to 900ml of distilled water. This solution was poured into a microwaveable container. The pH was then measured and HCl or NaOH was then added as necessary to make the solution have a pH of 6.

2.8.3.2 Antigen retrieval

After the dewaxing procedure, slides were placed into a glass jar containing 3% H_2O_2 in methanol (270ml methanol and 30ml H_2O_2) for 10 minutes. In a microwavable container 100ml citrate buffer (pH 6.0) was heated in the microwave for 12 minutes. Slides were transferred into a

plastic rack, rinsed in water then placed into the hot citrate buffer. The microwavable container was then closed and heated in the microwave for a further 10 minutes.

After 4 minutes in the microwave, the container was removed and the plastic rack of slides were placed under running water. Water was poured into a metal tray. Each slide was taken from the rack, excess water was drained off by blotting with paper and taking care not to disturb the tissue section. The margins of the tissue section were then marked using an Immedge pen, to aid viewing of the small section. The slide was then placed on the tray and the tissue section covered in horse serum, taking care to use enough volume to completely cover the section. The tray was then left for 5 minutes at room temperature.

2.8.3.3 Primary antibody incubation

The anti-IGFBP-3 antibody was then made up into a solution of 1 in 200 using phosphate buffer saline (PBS): 1µl antibody plus 200µl PBS. The horse serum was drained from all slides except the negative controls. The remaining samples were then covered fully in antibody solution and the lid placed on the metal tray to provide a humidified atmosphere. The tray was then placed in the incubator at 42°C for 1½ hours.

2.8.3.4 Secondary antibody incubation

The secondary antibody solution was made up of 2.35ml of PBS, 150µl of horse serum and 50µl of biotinylated secondary antibody. The solution was then vortexed.

The tray was then removed from the incubator. The slides were placed back into the rack and then into a glass jar containing PBS for 2 x 5 minutes to wash off excess antibody. The slides were then placed back

onto the metal tray, the edges dried and each tissue section was covered fully with the secondary antibody solution. The lid was then placed onto the tray and incubated at room temperature for 30 minutes.

2.8.3.5 Tertiary antibody incubation

The tertiary complex consisted of HRP- avidin and biotin complex which binds to the secondary antibody. The solution was vortexed and prepared at least 30 min before use.

The slides were then removed from the tray and washed twice in PBS for 5 min at a time. Excess PBS was blotted off the slide using tissue paper, taking care to avoid tissue section. The slides were then placed back on the metal tray and each tissue section was covered in tertiary solution. The tray was incubated at room temperature for 20 min.

After incubation with the tertiary complex, the slides were placed back into a rack and washed twice in PBS for 5 min. The slides were removed and a liquid DAB applied to each tissue section for 5 min. As soon as a brown colour develops, slides were washed in water.

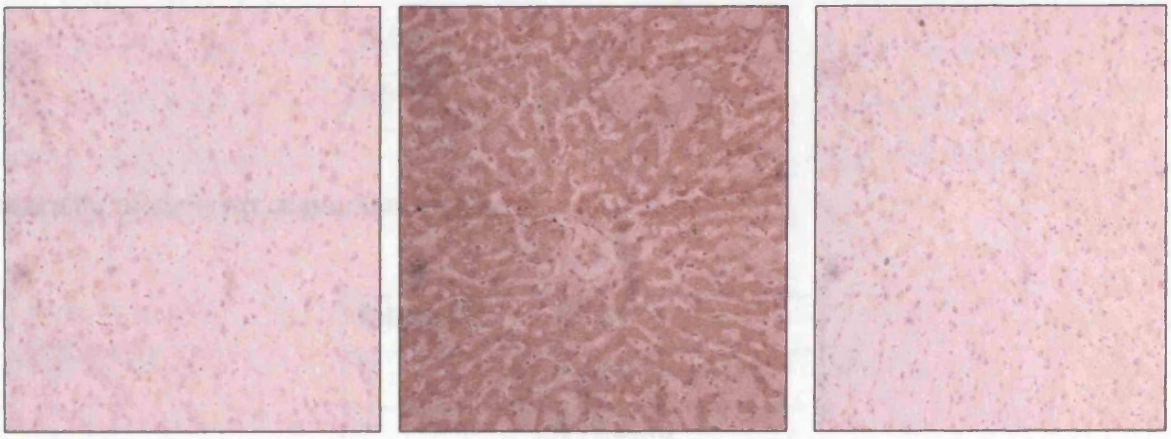
Each slide was then counterstained by rinsing in Gills haematoxylin for 30 sec followed by 1% acid-alcohol for 1 sec. Slides were then rinsed under running water for 2 min, 70% alcohol 2 min, 90% alcohol 2 min, 100% absolute alcohol for 2 min, in another absolute alcohol for 2 min, in xylene for 2 min and finally in another xylene for 2 min.

A coverslip was then quickly applied to each tissue section using Depex mounting medium and the slides were left overnight and viewed under the microscope the following day.

Sections of liver were used as a positive control in each run. The sections of normal breast and cancer were randomly mixed prior to staining.

Specificity was confirmed by complete absence of staining by pre-incubation of the antibody with IGFBP-3 ligand (Gropep, Australia) (Fig 7).

Figure 7. IGFBP-3 blocking peptide confirming specificity using sections of liver (1 in 200 dilution).



Negative control Positive control Blocking peptide

2.8.4 Quantification of Immunohistochemical Staining

Sections of normal and malignant breast tissue were randomly mixed prior to staining. Assessment was performed in a blinded manner on two separate occasions by a single investigator. In order to quantify IGFBP-3 protein expression, a scoring system based on the proportion of positive cells and intensity of staining was employed. This was scored as follows and a separate score determined for stromal and epithelial staining:

Proportion of positive staining cells:

No. Cells	Score
<5%	1
5-50%	2
>50%	3

Intensity of staining of positive cells:

Intensity	Score
0	1 (none)
+	2 (weak)
++	3 (moderate)
+++	4 (strong)

The two scores were added together to give a score out of 7. Separate scores were calculated for epithelial and stromal staining. A score of 1-3 was classified as negative and a score of 4-7 was positive. These two scores were combined together to give a total score out of 14: 1-6 =negative and 7-14 = positive with a subgroup of 10-14 = strongly positive. The procedure was repeated again without viewing the first set of results.

2.9 Serum IGF-I and IGFBP-3

2.9.1 Sample collection

5ml of blood was venesected from each patient undergoing breast cancer surgery involved in the study. Blood was taken from 36 patients on the day prior to breast surgery (pre-operative) and from 35 patients up to 6 months after surgery (post-operative). Of the post-operative group, patients were classified into those taking Tamoxifen (T) and those not taking Tamoxifen (NT).

The blood was collected aseptically into glass tubes containing no additives and care was taken to avoid needlestick injury. All samples were centrifuged at 10,000rpm for 15 minutes at room temperature. Serum was separated from red cells by pipetting and collected in a fresh, round bottomed eppendorf. These samples were frozen at -80°C.

2.9.2 Serum IGF-I

This was measured on all samples by an in-house radioimmunoassay, courtesy of the Growth Factor Lab, St Bartholomew's Hospital (249). Total IGF-I was measured after the samples were separated from their binding proteins by acetone extraction.

2.9.3 Serum IGFBP-3

Serum IGFBP-3 was measured using active IGFBP-3 Enzyme-Linked Immunosorbent Assay Kit. All samples and reagents were brought to room temperature before the assay.

Serum samples were thoroughly mixed prior to use and diluted to 1:100 in the standard dilutent containing 0ng/ml IGFBP-3 (in a protein based buffer). Wells to be used were then marked on the microtitration strips, all samples were performed in duplicate. 25µl of each standard (2, 5, 20, 50 and 100 ng/ml IGFBP-3), control (samples of high and low concentration IGFBP-3) and the unknown diluted samples were pipetted into each well. 50µl of assay buffer was then added to each well. The wells were then placed in a microplate shaker at approximately 700 rpm, for 2hours (room temperature). Each well was then aspirated and washed five times with wash solution. The microplate was then inverted and blotted dry on absorbent material.

The conjugate solution was then prepared by diluting the antibody-enzyme conjugate concentrate at a ratio of 1:50 using the assay buffer. 100µl of this solution was then added to each well. The microplate was then shaken again at 700 rpm for 1 hour at room temperature. Each well was then washed and aspirated a further five times followed by inverting the plate and blotting dry.

100µl of tetramethylbenzidine (TMB) solution was then added to each well and the plate then re-shaken at 700 rpm for 10 minutes at room temperature. 100µl of stopping solution (0.2M sulfuric acid) was then added to each well. The microplate was then placed in a microplate reader and absorbance measured at 450nm (background absorbance was set to 600nm).

2.10 In Vitro Tissue explant culture

2.10.1 Tissue collection

Fresh breast tissue samples were obtained from 6 breast cancer patients undergoing surgery. As before, full informed consent was obtained (Appendix A). All cancers were clinically palpable (>1-2cm in size).

2.10.2 Tissue processing

Each breast specimen was transported from surgery to the Department of Pathology immediately after removal from the body. The specimen was orientated, inked and a sample of tumour (T) and adjacent normal tissue (AN) excised by the Pathologist. AN was macroscopically normal tissue taken as far away from the T as possible. Care was taken when dissecting normal tissue to obtain glandular tissue and avoid fat. Each sample was then placed into seperate sterile cryogenic vials containing serum free DMEM and transported quickly to the laboratory.

2.10.2.1 Preparation of the medium

The stock solution used for all experiments contained ; 500ml Dulbecco's Modified Eagle's Medium (DMEM), 1g of bovine serum albumin, 5ml of penicillin/streptomycin mix (10mg/ml) and 0.5ml of amphotericin (0.25µg/ml). All were added to the medium via a sterile filter and stored at -4°C.

In order to carry out this culture technique, large tumours were necessary to provide enough tissue. The size of the specimen therefore was a limiting factor.

2.10.2.2 Preparation of IGFBP-3

The optimum concentration of IGFBP-3 for apoptotic effects was recommended at 200ng/ml. IGFBP-3 was prepared from 15µg lyophilized form. It was diluted in 75µl of 1mM hydrogen chloride (HCL) to make 75µl of 200µg/ml BP-3. This was aliquoted into 12µl vials to avoid freeze thaw cycles and stored at -80°C.

2.10.2.3 Preparation of Doxorubicin

For physiologically relevant cytotoxic effects in cell culture conditions, the recommended concentration of doxorubicin was 0.3µM. Doxorubicin was prepared from lyophilized form (MW=580). 1.16mg was carefully weighed and mixed thoroughly with 20ml medium to make a 10^{-4}M solution. This solution was then aliquoted into 1.2ml vials and stored at -80°C. For experimental use, one vial was thawed and vortexed. 1ml was added to 9ml of medium and vortexed to make a 10^{-5}M solution. 1ml of this solution was then added to a further 9ml of medium and vortexed to make a 10^{-6}M solution. 150µl of this solution was then added to 350ul of medium in order to make a 0.3µM solution,

2.10.3 Culture protocol

All experiments were carried out in a class 2 containment hood. The tissue was dissected into 1-2mg pieces using sterile scalpels and petri dishes. Each piece was washed in a separate petri dish containing medium prior to incubation in a 24 well plate at 37°C 5% CO_2 in the following conditions; medium, 200ng IGFBP-3, 0.3mM doxorubicin and a combination of 200ng IGFBP-3 and 0.3mM doxorubicin. The total volume in each well was 500µl. Doxorubicin is a cytotoxic agent used in the treatment of breast cancer. It causes apoptosis by interfering with microtubule formation. Explants were removed after 5 min, 24, 48 and 72 hours of incubation. The explants were immediately snap frozen in liquid nitrogen and stored at -80°C. The viability of the explant system was assessed by measuring LDH release and performing MTS assay on the medium at each time point.

2.10.4 Assessment of tissue explant system viability

2.10.4.1 MTS Cell proliferation assay

The MTS Cell Proliferation Assay was used to assess viability of the tissue explant technique. Cell viability can be reflected by the integrity of the mitochondria. When the MTS reagent (a tetrazolium salt) is applied to living cells, it is converted to a colour compound (formazan) with the emission of light at 490nm. The assay is based upon the reduction of the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS). NADPH/NADH produced by dehydrogenase enzymes in metabolically active cells bioreduce the MTS tetrazolium compound. This bioreduction results in a coloured product, soluble in tissue culture medium. Absorbance readings of medium and

the MTS dye were taken to determine the number of metabolically active cells in the breast tissue samples.

2.10.4.1 1 Protocol

A solution of 5 mg/ml MTS dissolved in PBS was made up and filter sterilised. 100µl of MTS dye was added to the tissue after incubation in 500µl media. All experiments were carried out in duplicate, in a 24 well plate. The plate was then incubated in a CO_2 incubator at 37°C for 4 hours. After 4 hours, 100µl of the media/dye mix was taken from the sample and control wells and an absorbance reading at 490nM wavelength was performed.

2.10.4.1.2 Controls

For each time frame, there was a corresponding well with medium only + explant and dye. This positive control was subjected to the same conditions as the other, experimental wells and was assayed with MTS in the same way.

Triton-X results in maximum disruption of plasma membranes and was used as a negative contol. To make up a solution of 2% Triton, 200µl of triton 100 was added to 9.8ml medium and mixed thoroughly. 500µl of this solution was then added to a well containing an explant and incubated as protocol.

2.10.4.1.3 Variability of the MTS assay

In order to look at the effect of the different tissue weights on the MTS absorbance, normal and malignant breast tissue was dissected into approximately 20 explants of visibly different sizes. These explants were

placed into a 24 well plate. MTS dye and medium were added to each sample well including 2 control wells. After 4 hours of incubation at 37°C 5% CO_2, the plate was removed from incubation. The tissue samples were removed, blotted dry using absorbant paper and weighed. The absorbances were read and MTS values adjusted according to their weights.

2.10.4.2 LDH Cytoxicity detection

The lactate dehydrogenase (LDH) assay is another colorimetric method which was used to evaluate cell death. It is based on the measurement of cytoplasmic enzyme activity that is released by damaged cells into cell culture medium upon damage to cellular plasma membranes. In contrast to the MTS assay which measures metabolic activity, this assay quantitates cell death and lysis. The amount of enzyme activity detected in the culture medium correlates to the proportion of lysed or dead cells (250).

A two step enzymatic reaction occurs when LDH containing tissue free media is incubated with the reaction mixture provided in the kit. The first reaction involves reduction of NAD+ to NADH/H+ by LDH catalysed conversion of lactate to pyruvate. In the second step, a catalyst from the reaction mixture transfers H/H+ from NADH/H+ to tertrazolium salt INT. This transfer results in the reduction of INT (yellow) to formazoan (red). The amount of red colour produced, therefore, correlates with the amount of formazoan formed and hence the amount of dead /membrane damaged cells in the medium. Absorbance may then be read spectrophotometrically.

This assay was performed on the culture medium for all six experiments.

2.10.4.2.1 Protocol

100µl of assay medium was added into duplicate wells of a 96 well plate. The reaction mixture was prepared immediately prior to the assay: mix 250µl of bottle 1 (catalyst) with 11.25ml of bottle 2 (INT dye solution). It was mixed thoroughly. 100µl of reaction mixture was then added to each well and incubated for 30min at 20-25°C. The plate was protected from light during this time. The 96 well plate was then placed into a microplate reader and the absorbance was read at 490nm.

2.10.4.2.2 Controls

A negative control of medium only was used for each time frame. A positive control of 2% Triton was also used for each time frame. Triton was used to ensure maximum LDH release for the purposes of a positive control.

2.10.4.2.3 Variability of the LDH assay

In order to assess the inter-assay variability of the LDH assay, duplicate sets of samples were used, where tissue availability allowed. Also, intra-assay variability was assessed by assaying each well 12 times.

2.10.5 Assessment of apoptosis

2.10.5.1 Introduction to apoptosis

Apoptosis is a mode of cell death used by multicellular organisms to

eradicate cells in diverse physiological and pathological settings (251). It is a normal component of the development and health of multicellular organisms (otherwise known as programmed cell death). It is characterised by cell shrinkage, chromatin compaction, plasma membrane blebbing and collapse of the cell into small intact fragments known as apoptotic bodies that are removed by phagocytes (252). This occurs in response to a variety of stimuli and does so in a controlled, regulated fashion. It is therefore distinct from cell death which occurs as a result of necrosis whereby uncontrolled cell death occurs and leads to lysis of cells, inflammatory responses and potentially serious sequelae.

2.10.5.2 Caspases

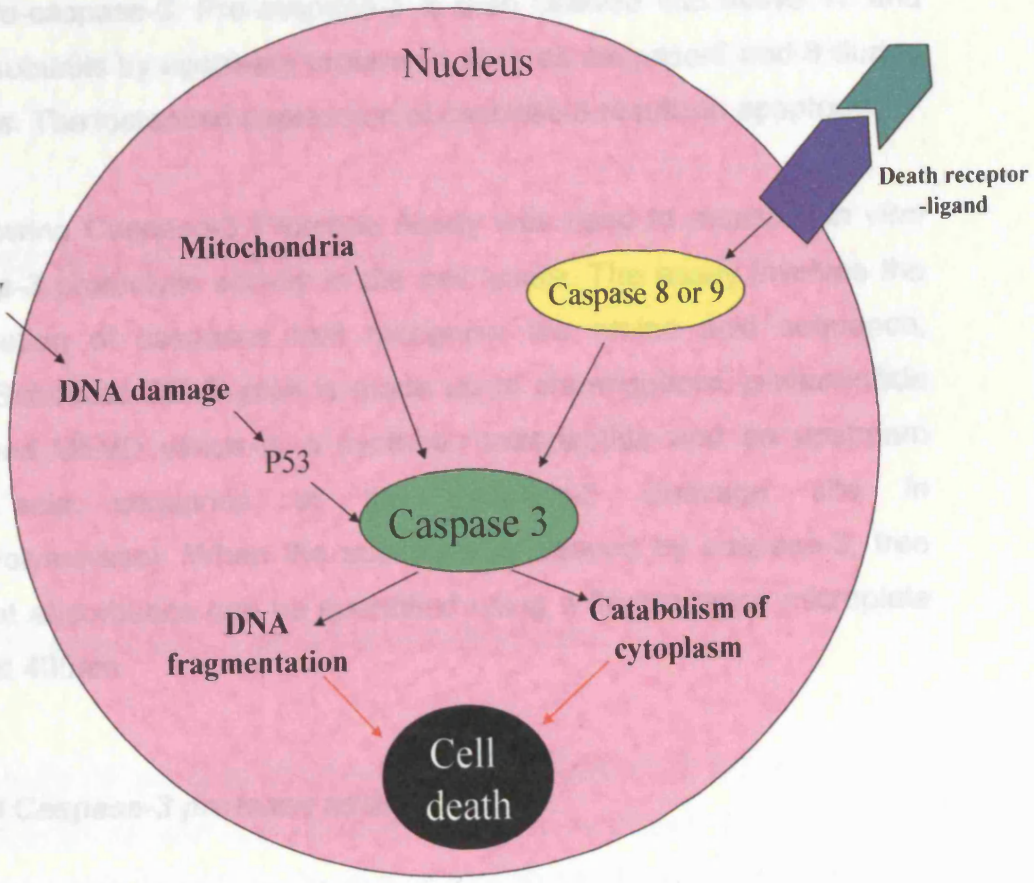
Caspases are a family of proteins that are typically activated in the early stages of apoptosis. They are a group of cysteine proteases that exist within the cell as inactive precursors (or zymogens). So far 14 mammalian caspases have been identified, some of which are involved in the regulation of apoptosis, the rest are involved in the processing of pro-inflammatory cytokines (253;254). They may be cleaved to form active enzymes following the induction of apoptosis. Once activated, they breakdown key cellular substrates which are necessary for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. They may also activate DNases which cleave the DNA in the nucleus. As a result, morphological changes occur.

Apoptosis may be induced by activation of death receptors. Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate caspases within seconds of ligand binding and so this mechanism is very rapid. They belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis. Death receptors include Fas, TNF receptor-1

and TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5. Other mechanisms of inducing apoptosis include cellular stress such as cytotoxic drugs, DNA damage and cytotoxic T lymphocytes which promote apoptosis by delivering a serine protease (granzyme B) which has a similar substrate specificity to caspases (Fig 8).

Induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis. Molecules that regulate caspases activation therefore play an important role in regulating cell survival.

Figure 8. Induction of apoptosis via the death receptor, granzyme B (via mitochondria) and cell injury.



2.10.5.2.1 Caspase-3

Caspase-3, otherwise known as CPP32 or apopain, is a key effector of the caspase family. It exists intra-cellularly as an inactive pro-enzyme called pro-caspase-3. Pro-caspase-3 is then cleaved into active 17 and 12 kDa subunits by upstream proteases such as caspase-6 and 8 during apoptosis. The increased expression of caspase-3 results in apoptosis.

The following Caspase-3 Protease Assay was used to measure *in vitro* caspases-3 proteolytic activity in the cell lysate. The assay involves the quantification of caspases that recognise the amino acid sequence, DEVD. Substrate DEVD-*p*NA is made up of chromophore, *p*-nitroanilide (*p*NA) and DEVD which is a synthetic tetrapeptide and an upstream amino acid sequence of the caspase-3 cleavage site in PARP(Polymerase). When the substrate is cleaved by caspase-3, free pNA light absorbance can be quantified using a fluorescence microplate reader at 405nm.

2.10.5.3 Caspase-3 protease assay

The frozen explants from the above six experiments were removed from -80°C storage and placed into liquid nitrogen. Each explant was then taken out of liquid nitrogen and homogenised in turn. Homogenisation was performed using a mortar, pestle and liquid nitrogen to grind the samples into a fine powder. The powder from each was then placed into separate sterile eppendorfs.150µl of chilled cell lysis buffer was then added (this contained tris buffered saline). The sample was then incubated on ice for 10 minutes. After 10 minutes, the samples were centrifuged for 1 minute at 10,000rpm. The cell lysate was then transferred to a 96 well plate. In a separate glass container, 50µl of 2 x

reaction buffer per sample was aliquoted. To this, 10µl of 1.0M DTT was added for every 1ml of 2 x reaction buffer used, immediately before use. 50µl of this solution was then added to each sample. 5µl of 4mM DEVD-*p*NA substrate was then added to each well and the plate was incubated in the dark at 37°C for 2 hours.

After 2 hours, the plate was transferred to a fluorescence microplate reader and the kinetics of the proteolytic cleavage of substrates was monitored using an excitation wavelength of 405nm.

2.10.6 Validation of caspase-3 assay

In order to validate the apoptotic findings of the caspase-3 protease assay, tissue explants were stained with anti-caspase-3 antibody and immunofluorescence.

2.10.6.1 Anti-caspase 3 immunohistochemistry

Anti-caspase 3 immunohistochemistry was performed on two of the above tumour explant experiments. Explants incubated for 24 and 48 were placed into 2ml of 4% paraformaldehyde fixative and stored at -4°C. Tissues were then embedded in paraffin and sectioned according to standard protocols (approximately 8-10 sections per explant). The standard procedure for dewaxing and rehydration of tissue slides was used, as described earlier. Each slide was stained with human anti-caspase-3 antibody at a dilution of 1 in 100, using the previously described protocol. The slides were randomly mixed prior to staining. Assessment of number of apoptotic cells was performed in a blind manner on two separate occasions by a single investigator. The mean number of apoptotic cells was calculated for all the slides prepared per explant.

2.10.6.2 Immunofluorescence

Immunofluorescence utilises the same protocol for immunohistochemistry as used before, however, detection of antigens involves fluorescence with fluorescent labeled antibodies. Tyramide Signal Amplification was used in combination with DAB for the anti-caspase 3 antibody staining on the tissue explant frozen sections from 2 experiments. TSA uses horseradish peroxidase to catalyze the deposition of a fluorophore-labeled tyramide amplification reagent onto tissue sections that have been previously blocked with proteins. The reaction takes less than 10 minutes and results in the deposition of numerous fluorophore labels immediately adjacent to the immobilised horseradish peroxidase enzyme. The fluorophores were then detected by fluorescence visualization techniques.

2.10.6.2.1 Protocol for Immunofluorescence

The protocol for immunofluorescence follows all the same steps as for DAB staining until the liquid DAB is added to each slide. A solution was made up of 450µl of amplification diluent and 9µl of fluorescein. This solution was then added to cover each tissue section. The slides were then left in the dark for 5 minutes and washed in PBS. Using fluorescence microscopy, the slides were then assessed for development of fluorescence and once ready, placed in PBS for 5 minutes. All slides were mounted using 'immunomount'.

3 Statistical Evaluations

All statistical calculations were carried out using Stats Direct (Ashwell, Herts, UK). P values <0.05 were considered significant.

3.1 RT-PCR data

All RT-PCR data was expressed as a copy number per µg total RNA. The median value was calculated for AN, T, NN, RN and RT for each marker. The differences in median copy number between tissue types for each marker were then calculated using non-parametric tests (Mann Whitney U).

In order to determine any correlation between markers, Spearman Rank correlation was used. In order to assess any correlation between IGFBP-3 mRNA expression and clinico-pathological variables, samples were divided according to each variable i.e. ER positive, ER negative, lymph node positive and lymph node negative. The difference in median copy numbers for each variable was then calculated using non-parametric test, Mann Whitney U.

3.2 BP-3 protein expression

The differences in staining between the normal breast tissue versus tumour and stromal versus epithelial staining were evaluated using a two sided Fisher exact test or a chi-square test as appropriate. These findings were also correlated to the following clinicopathological parameters; tumour size (greater or less than 2cm), tumour histology, nodal status,

vascular invasion and oestrogen receptor status using Spearman rank correlation.

3.3 Serum IGF-I and BP-3 levels

In order to calculate differences in serum IGF-I and BP-3 levels in pre and post-operative breast cancer patients, Mann Whitney U non-parametric tests were used. For determining the IGF-I BP-3 ratios for each patient, each value was first corrected for age and then ratios were obtained and compared again using Mann Whitney U.

3.4 Tissue explant culture

For MTS, LDH and caspase-3 assays absorbance readings were recorded and mean values and standard errors of the mean were calculated. The differences were then compared using Mann Whitney U.

3.5 Anti-caspase 3 immunhistochemistry

For each sample, sections were randomly mixed and number of apoptotic cells were counted in a blinded manner by a single observer. Mean values for each sample were then calculated and differences assessed using Mann Whitney U.

4. Results

4.1 Results of RNA Quantification

4.1.1 Ribogreen standard curve results

Fig 9A demonstrates the fluorescence values for four normal and four malignant breast samples analysed using the Ribogreen method. Each value is a mean of three values. The RNA concentration for each sample was then calculated using the y-intercept value from the standard curve (Fig 9B) and the dilution factor as previously mentioned. The Ribogreen method is particularly useful for detecting smaller concentrations of RNA. For example, it can be seen that normal breast tissue produces approximately 3-4 times less RNA than breast tumours. This technique was therefore used to detect the smaller quantities of RNA extracted from normal breast tissue. Unfortunately, unlike the Agilent Bioanalyzer, this technique only provides information on quantity and not quality of RNA.

4.1.2 Results of Agilent Bioanalyzer

For each lab chip analysed, the well marked ladder should produce 7 clear peaks in order to confirm that the gel has worked (Fig 10). Fig 11 shows an electropherogram and an autoradiograph of extracted RNA from a breast sample using the Agilent Bioanalyzer. The electropherogram shows three peaks. The first peak is a marker that enables comparison between samples. The following two peaks represent the two ribosomal peaks. The ratio of these peaks should be approximately 2:1 (28S:18S).This ratio in conjunction with a smooth

baseline represents good quality RNA. RNA degradation is seen with a decreasing ratio of ribosomal bands, additional peaks and a decrease in the RNA signal or shorter fragments. The height of the peaks should also compare well with the marker peak thereby indicating a good concentration of RNA. The Agilent software is then able to calculate the ratio of the sample peaks to the marker peak thereby providing the RNA concentration for each sample.

Figure 9 A and B. RNA quantified using Ribogreen(A). The standard curve(B) shows that increasing fluorescence correlates well with increasing RNA concentration.

A

Sample no	Mean fluorescence	RNA ug/ul
260N	4120	20.1
263N	3509	25.8
217N	1663.3	10.1
278N	1963.3	12.3
266T	3359.6	75.3
267T	3589.3	81
217T	2707.6	59.3
278T	3402.3	76.3

B

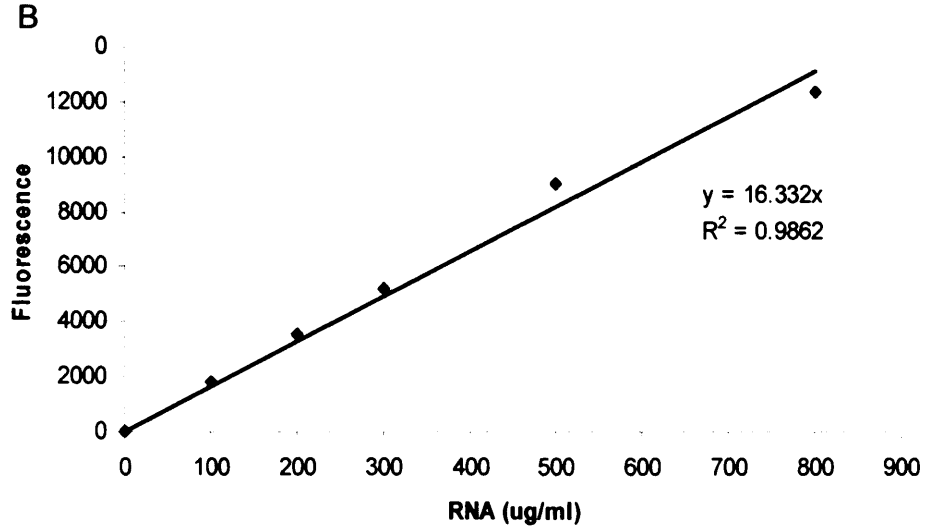


Figure 10. Representative electropherogram (A) and autoradiograph (B) of a single RNA sample. The ladder demonstrates 6 RNA peaks and 1 marker peak. The marker allows comparison with other chips.

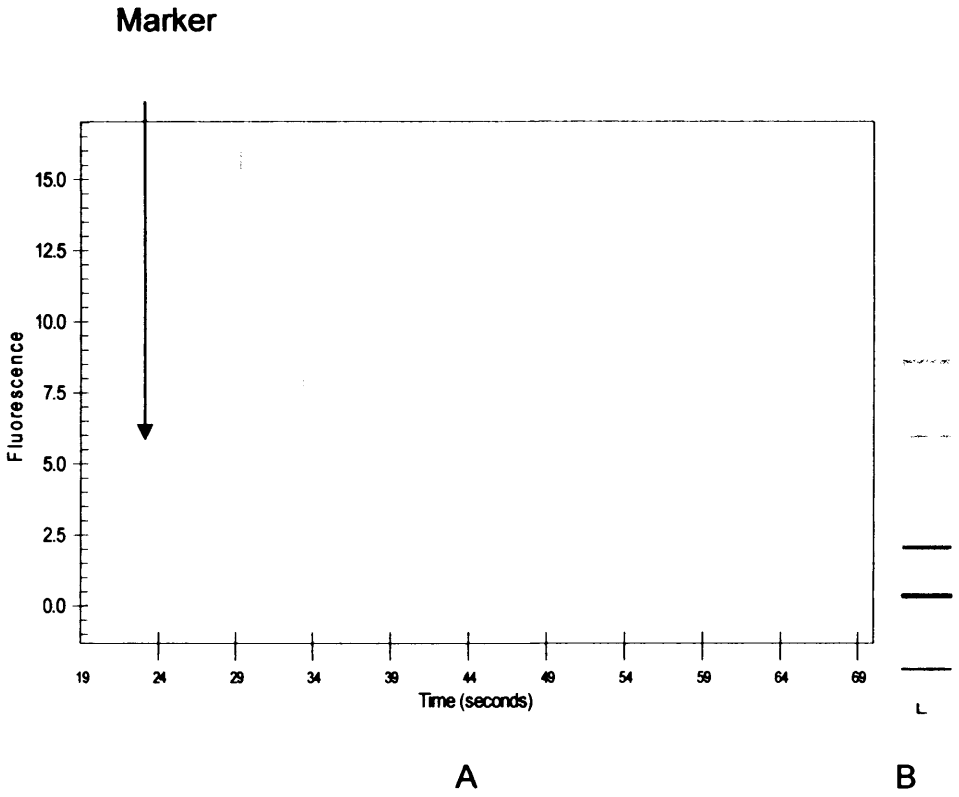
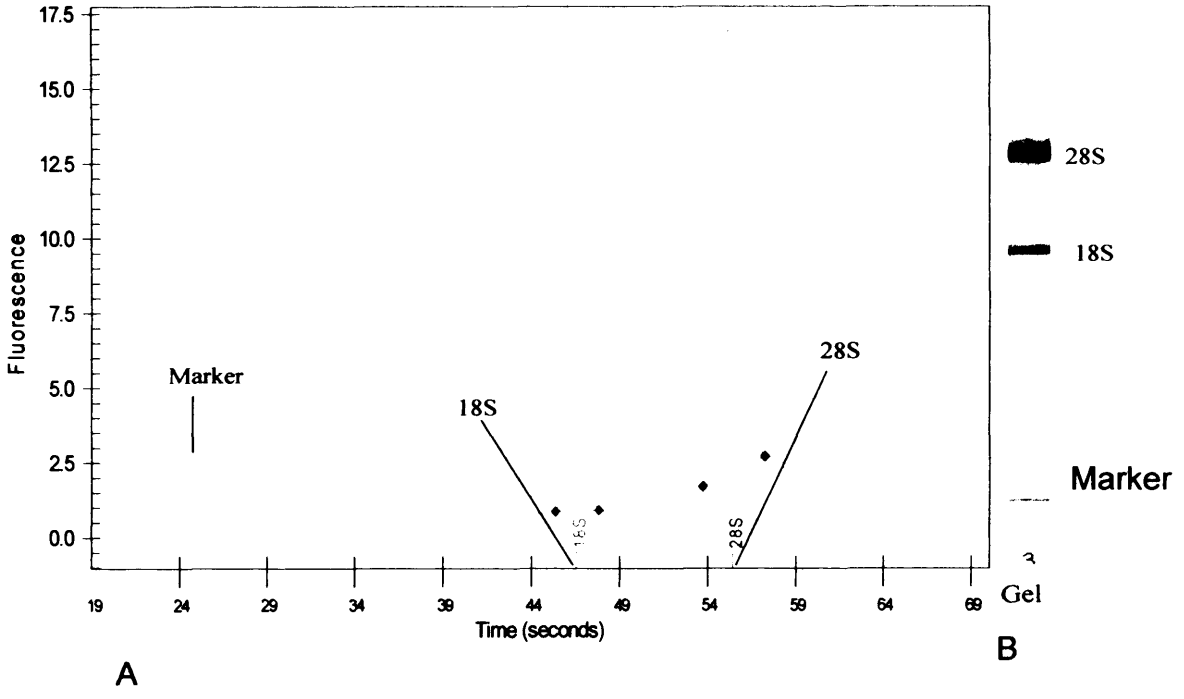


Figure 11. Electropherogram (A) and autoradiograph (B) of extracted RNA demonstrating high quality and quantity of total RNA. Two ribosomal peaks are seen with a ration of 2:1.



4.2 Results from RT-PCR

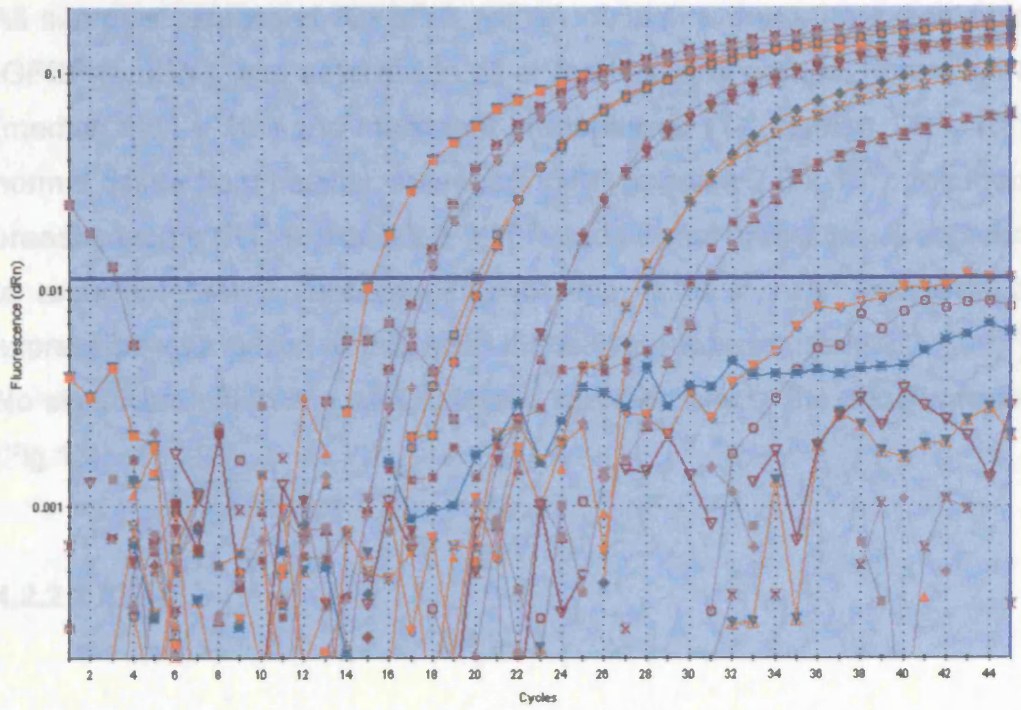
4.2.1 Results from generation of standard curve from amplicons

A linear graph was constructed for each amplicon, the high and low C_t values were discarded to correct for pipetting errors and the average of the remaining values was calculated. The C_t value is inversely proportional to the log of the initial copy number (255). Standard curves were constructed by plotting the C_t values with 95% confidence intervals against the logarithm of the initial copy numbers. The copy numbers for each of the experimental samples were then calculated after real-time amplification from the linear regression of that standard curve.

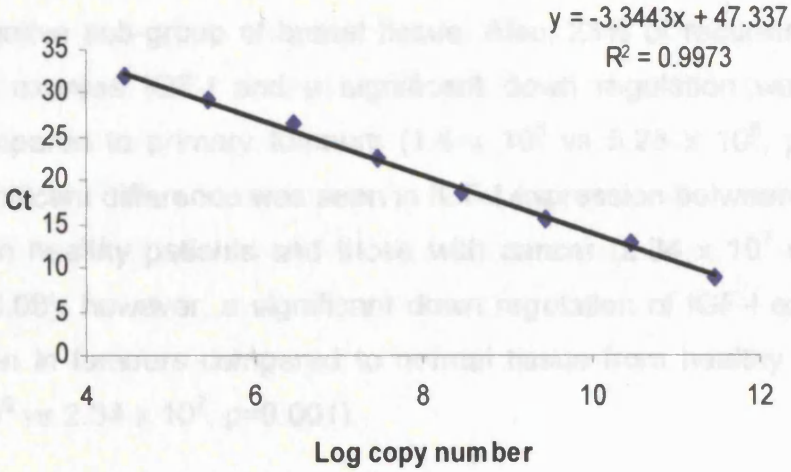
Fig (12) demonstrates the amplification plot of IGFBP-3 amplicon. Serial dilutions of the BP-3 amplicon were diluted as previously described. Fluorescence values were recorded for each known concentration and a standard curve (Fig B) was constructed of C_t values against log copy number. Fig A demonstrates smooth exponential amplification of the amplicon. The run was repeated if successful amplification did not occur.

Figure 12. Amplification plot of BP-3 amplicon (A) and construction of standard curve (B). Serial dilutions result in different fluorescence values. The smaller the Ct value, the higher the concentration of DNA.

A



B. Standard curve of BP-3 amplicon. Each value is the mean of three experiments, each sample was performed in duplicate.



4.2.2 Results from individual markers

4.2.2.1 IGFBP-3

All samples expressed IGFBP-3 mRNA. A high universal expression of IGFBP-3 mRNA was detected in all of the following groups; normal (AN) (median 1.61 x 10^8) and malignant breast tissue (T) (median 1.6 x 10^8), normal tissue from healthy volunteers (NN) (median 2.6 x 10^8), recurrent breast cancers (RT) (median 1.2 x 10^8) and normal breast tissue adjacent to recurrent breast cancers (RN) (median 1.20 x 10^8). The level of expression was similar to that seen in the liver (approximately 1.0 x 10^{10}). No significant difference was detected between any of the above groups (Fig 13).

4.2.2.2 IGF-I

A significant down regulation of IGF-I mRNA was seen in breast tumours when compared to adjacent normal breast tissue, (5.28 x 10^6 vs 1.18 x 10^7, p=0.003) (Fig 14). Interestingly, 5 tumours (9%) and 3 adjacent normal (6%) samples did not express IGF-I at all indicating an IGF-I negative sub-group of breast tissue. Also, 23% of recurrent tumours did not express IGF-I and a significant down regulation was seen when compared to primary tumours (1.4 x 10^6 vs 5.28 x 10^6, p=0.0001). No significant difference was seen in IGF-I expression between normal tissue from healthy patients and those with cancer (2.34 x 10^7 vs 1.18 x 10^7, p=0.08), however, a significant down regulation of IGF-I expression was seen in tumours compared to normal tissue from healthy patients (5.28 x10^6 vs 2.34 x 10^7, p=0.001).

4.2.2.3 VEGF

All samples expressed VEGF mRNA. No statistical differences in level of expression can be seen amongst the groups of breast tissue (Fig 15). There was also a large variation in expression seen in primary breast tumours.

4.2.2.4 COX-2

All samples expressed COX-2 mRNA (Fig 16). A significant up regulation was seen in the normal tissue adjacent to cancer (AN) when compared to breast tumours (T) (median copy number 3.14×10^7 vs 1.15×10^7, p=0.0001) and normal tissue from healthy patients (p=0.004). No difference in expression was seen between recurrent tumours (RT) (2.37×10^7, range $4.72 \times 10^6 - 8.1 \times 10^7$), their adjacent normal tissue (RN) (1.28×10^7, range $1.36 \times 10^6 - 5.1 \times 10^7$)and also with primary tumours (T).

4.2.2.5 1 alpha hydroxylase

Median 1αOHase mRNA levels were significantly lower in the AN samples (1.7×10^7; range $1.04 \times 10^5 - 1.57 \times 10^8$) compared with NN samples (5.2×10^7; range $4.59 \times 10^6 - 2.52 \times 10^8$; $p<0.01$) (Fig 17). Median mRNA copy number in T samples (2.8 x 107; range $1.75 \times 10^6 - 3.38 \times 10^8$) was higher than in paired AN samples ($p<0.04$) with higher levels (>3 fold) detected in 11/30 tumours, and lower levels in 4 tumour samples .

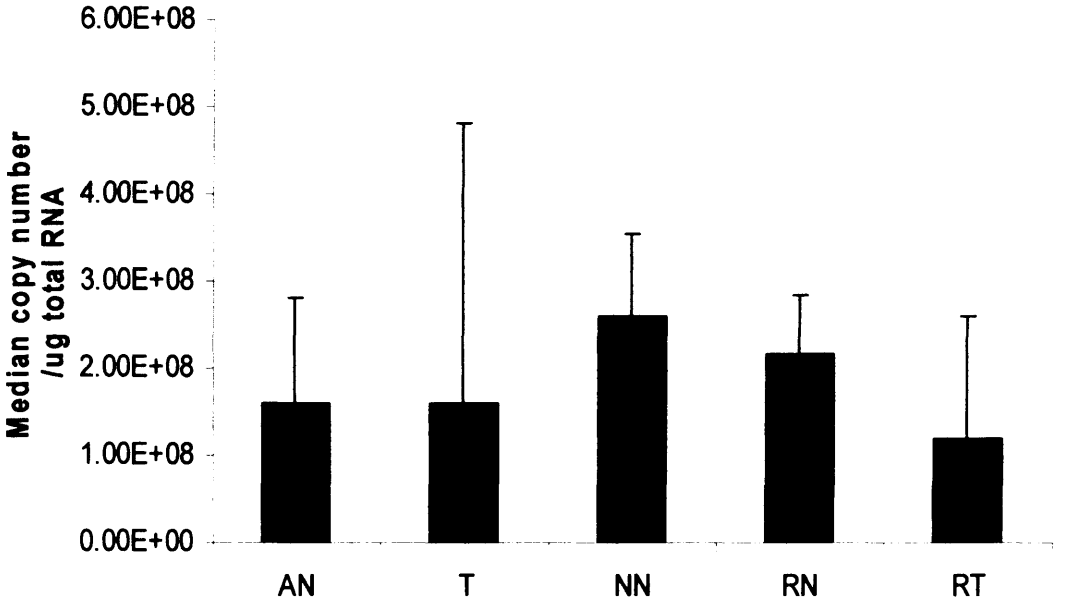
4.2.2.6 Vitamin D receptor

Median VDR mRNA levels were significantly higher in tumours (3.3×10^6; range $7.12 \times 10^4 - 8.7 \times 10^7$) compared to both AN ($1.65 \times 10^6$; range $5.21 \times 10^5 - 8.45 \times 10^6$; p=0.002) and NN samples (median 1.55×10^6; range $4.83 \times 10^5 - 7.19 \times 10^6$; p=0.01) (Fig 18). There was no difference between AN and NN samples.

4.2.2.7 24 hydroxylase

Median 24OHase mRNA levels were significantly higher in healthy normal breast tissue (NN) (1.0×10^8, range $1.1 \times 10^7 - 6.1 \times 10^8$) compared to both AN ($9.7 \times 10^6$, range $2.6 \times 10^5 - 2.9 \times 10^8$, p=0.0001) and tumours (1.3×10^7, range $3.4 \times 10^5 - 5.3 \times 10^8$, p=0.002) (Fig 19). There also appeared to be a down regulation of 24Ohase in recurrent breast cancers (RT, RN).

Figure 13. The expression of IGFBP-3 mRNA in breast tumours (T), adjacent non-cancerous tissue (AN), normal tissue from healthy patients (NN), and recurrent normal (RN) and recurrent malignant (RT) tissue. Values are the mean and standard error of the mean (SEM).

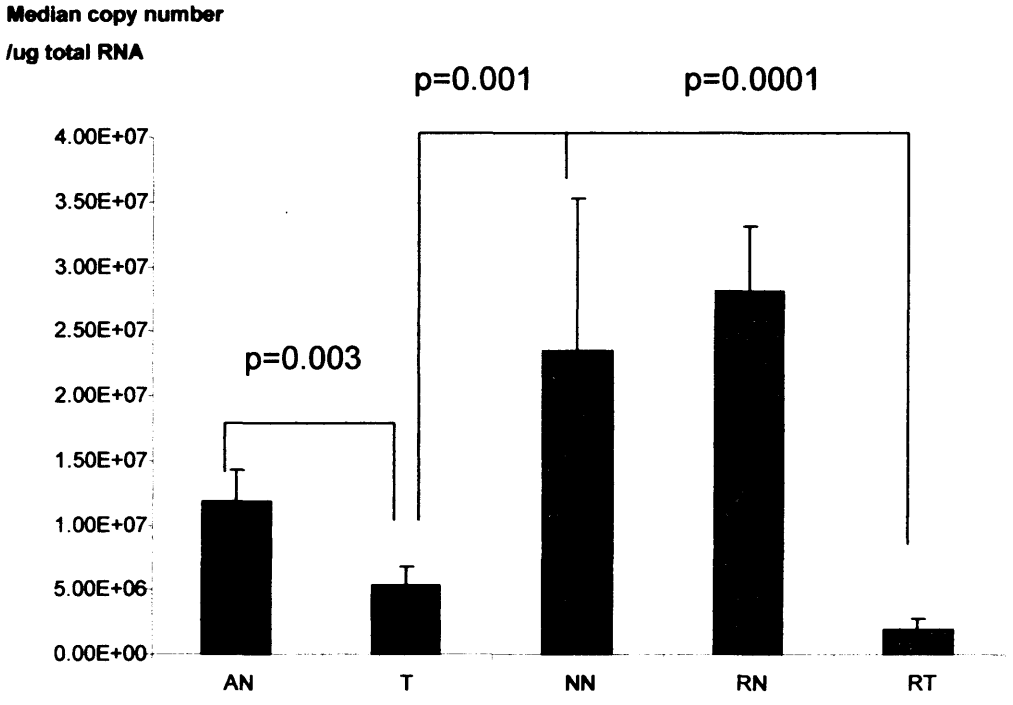


AN & T n=53

NN n=17

RN & RT n=10

Figure 14. The mRNA expression of IGF-I demonstrating down regulation in tumours (T) compared to adjacent normal tissue (AN). Values are expressed as mean + SEM.

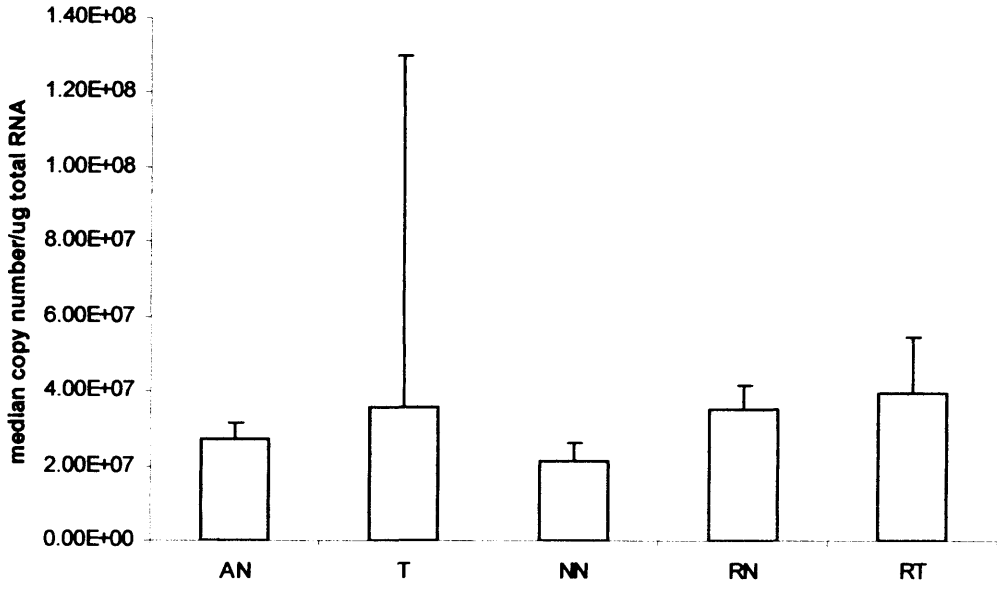


AN & T n=51

NN n=17

RN & RT n =10

Figure 15. VEGF mRNA expression showing similar levels of expression in all types of breast tissue. Values are expressed as mean + SEM.

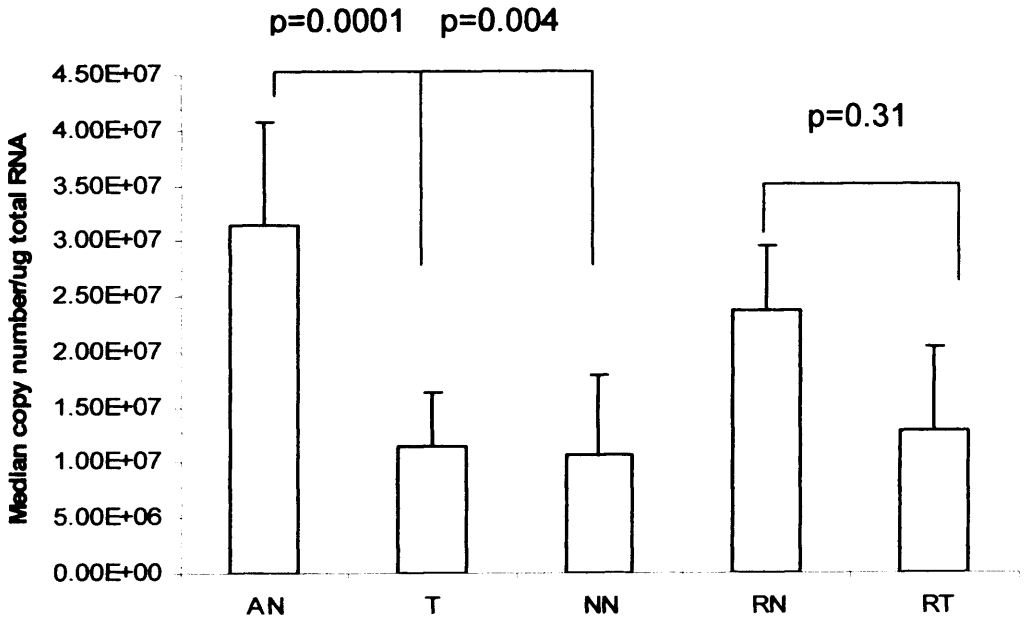


AN & T n=38

NN n=17

RN & RT n=10

Figure 16. Expression of COX-2 mRNA. An up regulation is seen in adjacent normal tissue (AN) when compared to breast tumours (T) and normal tissue from healthy patients (NN). Values are expressed as mean + SEM.

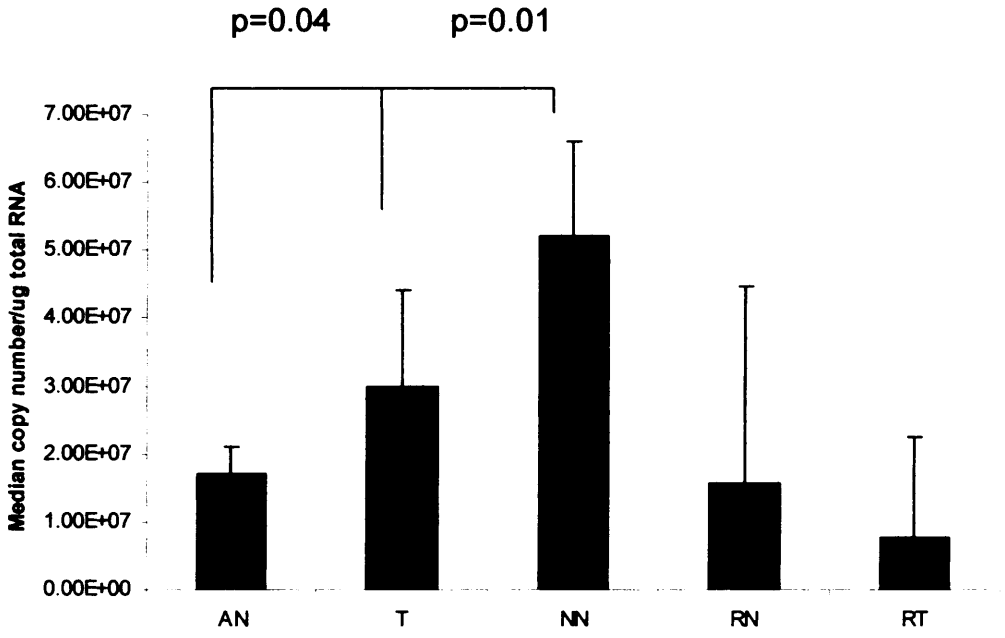


AN & T n=47

NN n=17

RN & RT n=10

Figure 17. Expression of 1αOHase mRNA. A down regulation can be seen in T and AN when compared to normal tissue from healthy patients (NN). Values are expressed as mean + SEM.

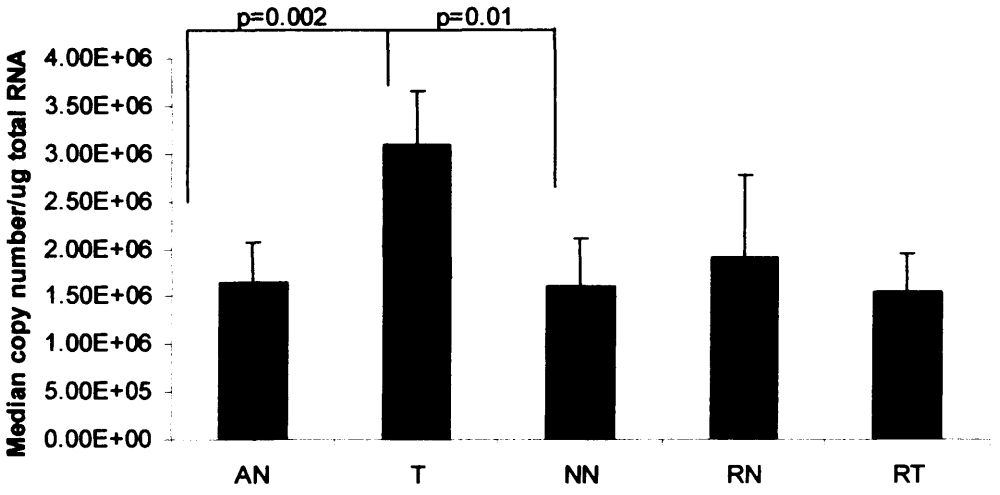


AN & T n=35

NN n=17

RT & T n=10

Figure 18. Expression of VDR mRNA. A significant up regulation can be seen in T when compared to AN and NN. Values are expressed as mean + SEM.

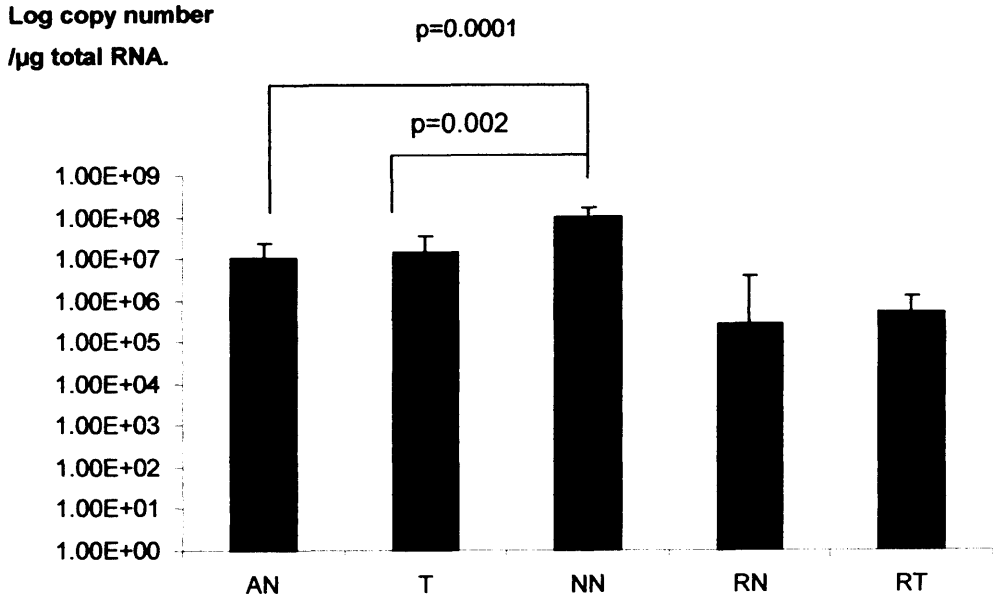


AN & T n=24

NN n=17

RN & RT n=10

Figure 19. Expression of 24 OHase mRNA. A significant down regulation is seen in AN and T when compared to NN. Values are expressed as mean + SEM (n=24).



AN & T n=24

NN n=17

RN & RT n=10

4.2.3. Results from correlation with clinico-pathological features

The breast tumour samples were divided into two groups; those expressing high (> 1.0 x 10^8, n=35) and low levels of IGFBP-3 (<1.0 x 10^8, n=18) mRNA for further analysis. A statistically significant increase in ductal carcinoma in situ (DCIS), known precursor for invasive breast cancer, was found in the high group when compared to the low group ($p<0.0001$, Mann Whitney U). On further analysis, there was also found to be a statistically significant increase in the presence of extensive high grade DCIS between the two groups ($p<0.0008$, Mann Whitney U). The presence of extensive, high grade DCIS is thought to predict for a substantially increased risk of axillary node involvement and systemic disease with a tendency for poor tumour markers (256).

No correlation was found between IGFBP-3 mRNA expression and the following clinico-pathological parameters: tumour size, tumour grade, histological type, ER/PgR status, lymph node status and presence of vascular invasion.

4.2.4. Results of correlation of BP-3 with other tumour associated genes.

A positive correlation was detected between IGF-I and IGFBP-3 mRNA in breast tumours (Spearman rank, R=0.38, p=0.01).No significant correlation was detected between IGFBP-3 and IGF-I mRNA in normal breast tissue. Similarly, no significant correlation was detected between IGFBP-3 mRNA and tumour associated genes; VEGF and COX-2 (Table

3). A positive correlation was detected, however, between IGFBP-3 levels in normal breast tissue and housekeeping gene, GAPDH (R=0.4, p=0.02, Spearman Rank correlation). No such correlation was seen with breast tumours, thereby confirming its variable expression in normal and cancerous tissue. Within the breast cancers, GAPDH expression ranged from 5.28×10^7 to 5.88×10^{10} and therefore supports the evidence that it is not a reliable internal reference for normalisation of PCR assays.

A positive correlation was detected between IGFBP-3 and 1 alpha hydroxylase mRNA expression in breast tumours (R=0.4, p=0.01, Spearman Rank Correlation). No other correlation was detected between IGFBP-3 and other members of the vitamin D axis; vitamin D receptor (VDR) and 24 hydroxylase, the enzyme responsible for breaking down active vitamin D.

Table 3. Correlations between IGFBP-3 mRNA from breast tumours with other markers. A positive correlation is seen with IGF-I and 1αOHase.

Marker	Correlation coefficient (R)	P value
IGF-I	0.37	0.01
VEGF	0.2	0.19
COX-2	-0.11	0.23
GAPDH	-0.05	0.7
1-alpha hydroxylase	0.4	0.01
Vitamin D receptor	0.2	0.2
24 hydroxylase	0.04	0.85

4.2.5. BP-3 expression and survival

By the time of writing, 4 deaths had occurred as a result of breast cancer out of the cohort of 53 women (mean follow up of 20 months). All four of these tumours expressed high levels of IGFBP-3 mRNA and their tumours were associated with extensive high grade DCIS, however, this did not reach statistical significance.

4.3. Results from Immunohistochemistry

4.3.1. Results of DAB staining

Immunohistochemical analysis of IGFBP-3 in normal and malignant breast tissue demonstrated positive immunoreactivity in 96% samples (46 out of 48) (Table 4). Two samples consisting of one tumour and one normal sample were negative (4%). 24 samples (50%) were classed as strongly positive and 22 (46%) were weakly positive. No difference in immunostaining was seen between normal and malignant breast tissue. No statistically significant correlation was seen between protein expression and IGFBP-3 mRNA (R= -0.2, p=0.17).

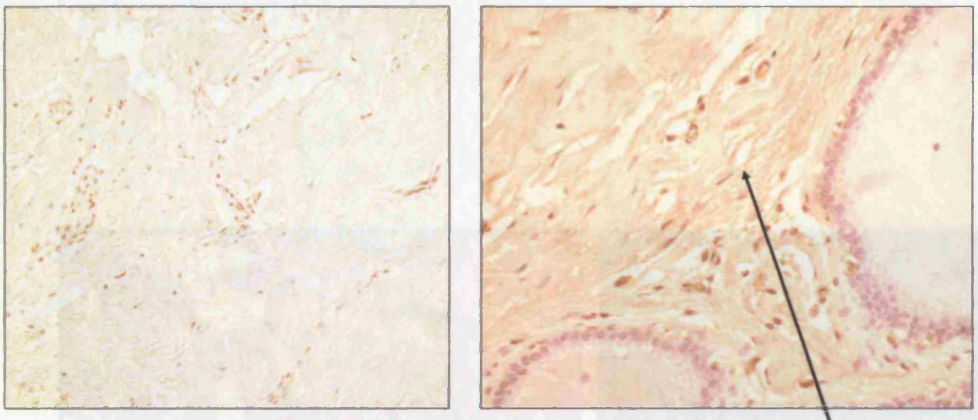
Table 4. Immunostaining of IGFBP-3 in normal (N) and malignant (T) breast tissue demonstrating that 96% of samples stained positive for BP-3 and 50% of these samples were strongly positive.

	N	T	Total
Negative (1-6)	1 (4%)	1 (4%)	2 (4%)
Positive (7-9)	9 (38%)	13 (54%)	22 (46%)
Strongly positive (10-14)	14 (58%)	10 (42%)	24 (50%)
Total	24	24	48

4.3.1.1 Stromal vs epithelial staining

There was an increase in stromal versus epithelial staining in normal and tumours (Fig 20, 21 and 22). In the normal breast samples, 67% of samples (16 out of 24) stained positively in the epithelium whereas 100% stained positively in the stroma ($p<0.0001$) (Table 5). Similarly in breast tumours, 46% of samples stained positively in the epithelium as opposed to 100% stromal staining ($p<0.0001$) (Table 6). Overall, there appeared to be a similar pattern of staining for both normal and tumour sections, although more tumours than normals stained negative in the epithelium (54% vs 33%).

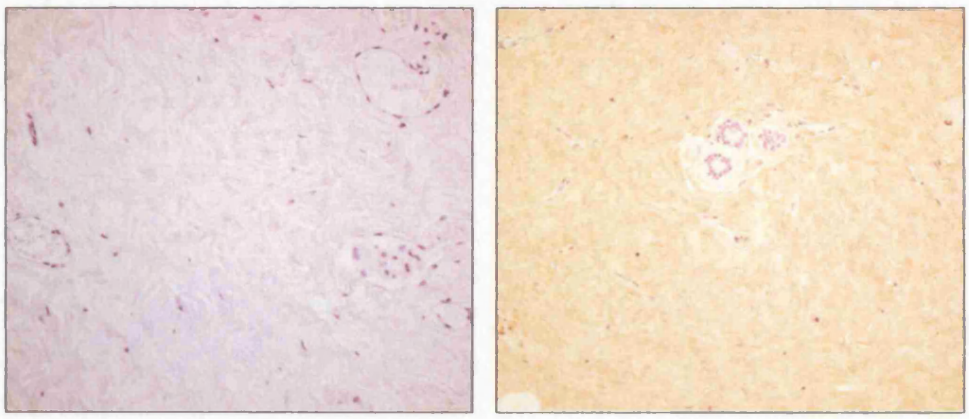
Figure 20. IGFBP-3 peptide is detected in the stroma of a breast tumour expressing BP-3 mRNA.



Negative control

Positive staining, stromal

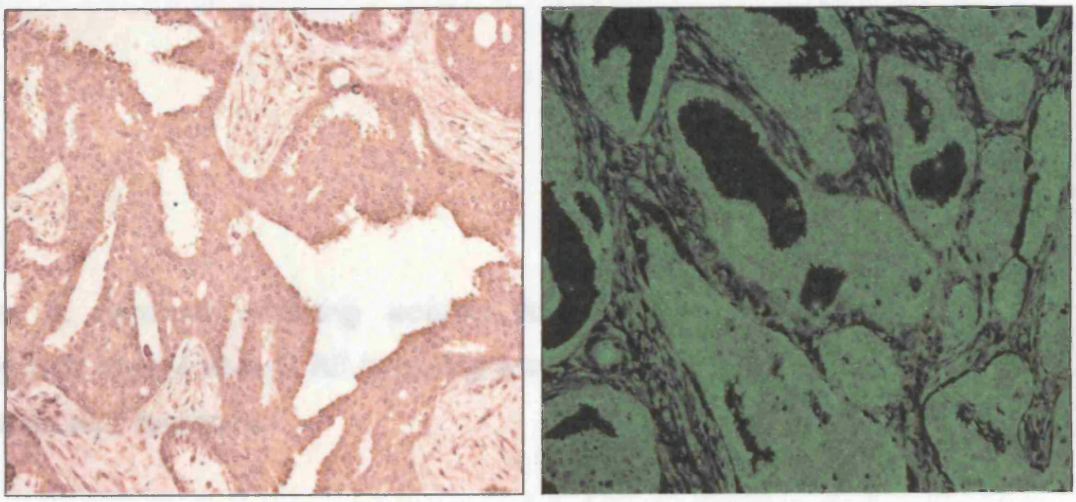
Figure 21. IGFBP-3 staining of normal breast tissue demonstrating stromal versus epithelial expression.



Negative control

Normal breast tissue

Figure 22. IGFBP-3 protein expression in a breast tumour demonstrating strong epithelial and stromal staining. Immunofluorescence confirms findings.



DAB staining Immunofluorescence

Table 5. Epithelial staining scores for IGFBP-3 in normal and malignant breast tissue. Approximately half of all samples are negative for IGFBP-3, more so in tumours when compared to normals (54% vs 33%).

	N	T	Total
Negative(1-3)	8(33%)	13(54%)	21(44%)
Positive(4-7)	16(67%)	11(46%)	27(56%)
Total	24	24	48

Table 6. Stromal staining scores for IGFBP-3 in normal and malignant breast tissue. All samples stain positively for BP-3 in both normals and tumours.

	N	T	Total
Negative(1-3)	0	0	0
Positive(4-7)	24(100%)	24(100%)	24(100%)
Total	24	24	48

4.3.2. Correlation with Clinicopathological Parameters

A statistically significant correlation was detected between IGFBP-3 negative tumours and lymph node negativity ($p=0.05$, Fisher's Exact test). No significant correlation was observed, however, between IGFBP-3 protein expression and tumour size (greater or less than 2cm), tumour histology, vascular invasion, oestrogen receptor status and age (Table 7).

Table 7. Correlation between tumour IGFBP-3 protein and clinico-pathological parameters.

	Number of patients (%)			
Parameter	All patients (%)	IGFBP-3 negative/weakly positive (score1-9)	IGFBP-3 strongly positive (score10-14)	P-value (Fisher's exact test)
Histology:				
Ductal	12(50)	6(50)	6(50)	0.6
Lobular	2(8.3)	1(50	1(50)	
Other	10(41.7)	6(60)	4(40)	
Tumour size:				
< 20mm	13(54)	8(62)	5(38)	0.46
≥ 20mm	11(46)	5(45.5)	6(54.4)	
Nodal status:				
0	11(46)	7(64)	4(36)	0.05
<5	8(33)	4(50)	4(50)	0.6
>5	4(21)	2(50)	2(50)	
Grade:				
1	3(12.5)	2(66.6)	1(33.3)	0.59
2	11(45.8)	5(45.5)	6(64.5)	
3	10(41.6)	6(60)	4(40)	0.8
Vacular invasion:				
Not detected	14(58)	9(64)	5(36)	0.27
positive	10(42)	4(40)	6(60)	
ER/PgR negative	4(17)	1(25)	3(75)	0.2
ER/PgR positive	19(83)	11(58)	8(42)	
DCIS:				
No	8(33.3)	6(75)	2(25)	0.18
Yes	16(66.6)	7(44)	9(56)	
<60yrs	12(50)	5(41.6)	7(58.3)	0.4
≥60yrs	12(50)	7(58.3)	5(41.6)	

4.4. Results of serum IGF-I and IGFBP-3

4.4.1. Serum IGF-I

In the pre-operative group, the mean patient age was 61 years and the average IGF-I was 162ng/ml (range 100 – 425ng/ml). In the post-operative group, the mean patient age was 64 years and the average IGF-I was 142ng/ml (range 56 – 257ng/ml). Within the post-operative group, 28 (80%) patients were taking tamoxifen (T) and 7 (20%) were not (NT). The mean age of patients in NT was 62 years and the average IGF-I was 153ng/ml (range 79-257ng/ml). In the T group, the patients had a mean age of 55 years which appeared much younger than NT, however, this was not statistically significant (Mann Whitney, $p=0.1$) probably as a result of a small sample size. The average serum IGF-I in this group was 130ng/ml (range 59-197ng/ml)

In order to account for the age related influence in serum IGF-I levels, a ratio of the serum value divided by the upper limit of the age related normal range was calculated (Tables 8 and 9). With this correction, serum IGF-I levels were decreased in the post-operative patients taking tamoxifen when compared to pre-operative patients and post-operative patients not taking tamoxifen (Mann Whitney, $p<0.05$).

Table 8. Average serum values of IGF-I according to age.

Age (years)	Average serum IGF-I concentration (ng/ml)
19-29	125 - 329
30 -39	120 -330
40 - 49	136 - 369
50 - 59	108 - 263
60 - 70	108 - 229

Table 9. Serum IGF-I levels expressed as a ratio of the upper limit of the age related normal range.

	Median ratio +/- standard deviation
Pre-operative	0.6 +/- 0.18 *
Post-operative NT	0.62 +/- 0.14 *
Post-operative T	0.52 +/- 0.14

* = $p<0.05$ (Mann Whitney U), when compared to post-operative T.

4.4.2. IGFBP-3

Serum IGFBP-3 levels increase from late childhood to puberty and then gradually fall during adult life. The levels are also higher in females than in males (normal range 2.6 – 5.5mg/l versus 1.5 – 4.6mg/l).

In the pre-operative group, the mean patient age was 61 years and the average IGFBP-3 level was 3mg/L (range 1.6 – 5.5mg/L). In the post-operative group, the mean patient age was 64 years and the average IGFBP-3 was 3.1mg/L (range 1.3 – 5.3mg/L). Within this post-operative group, 28 (80%) patients were taking tamoxifen (T) and 7 (20%) were not (NT). The average age of the NT group was 62 years and the average IGFBP-3 was 3.3mg/L (range 1.3 5.3mg/L). The average age of the T group was 55 years with a mean IGFBP-3 of 3.2mg/L (range 1.4 – 5.2mg/L). Tamoxifen clearly has no effect on serum IGFBP-3 levels.

4.4.3. IGF-I and IGFBP-3 serum levels

The IGF-I:BP-3 ratio was determined for the three groups of patients; pre-operative, post-operative NT and post-operative T. To account for the age related decline of IGF-I, the IGF-I values were calculated as a ratio of the upper limit of the age-related normal range (Table 10). IGFBP-3 values were also calculated as the ratio of the upper limit of the normal range.

The results show that the IGF-I:BP-3 ratio is significantly higher in the pre-operative group than the post-operative T group (Mann Whitney, p=0.05). There is no significant difference, however, between the pre-operative group and the post-operative group not taking Tamoxifen.

Table 10. Serum IGF-I BP-3 ratio expressed as a ratio of the upper limit of the normal range.

	Median ratio +/- standard deviation
Pre-operative	0.051 +/- 0.027 *
Post-operative NT	0.054 +/- 0.03 **
Post-operative T	0.04 +/- 0.013

* p=0.05, when compared to post-operative T

** p=0.09, when compared to post-operative T

4.5 Results of viability assessment of culture breast explants

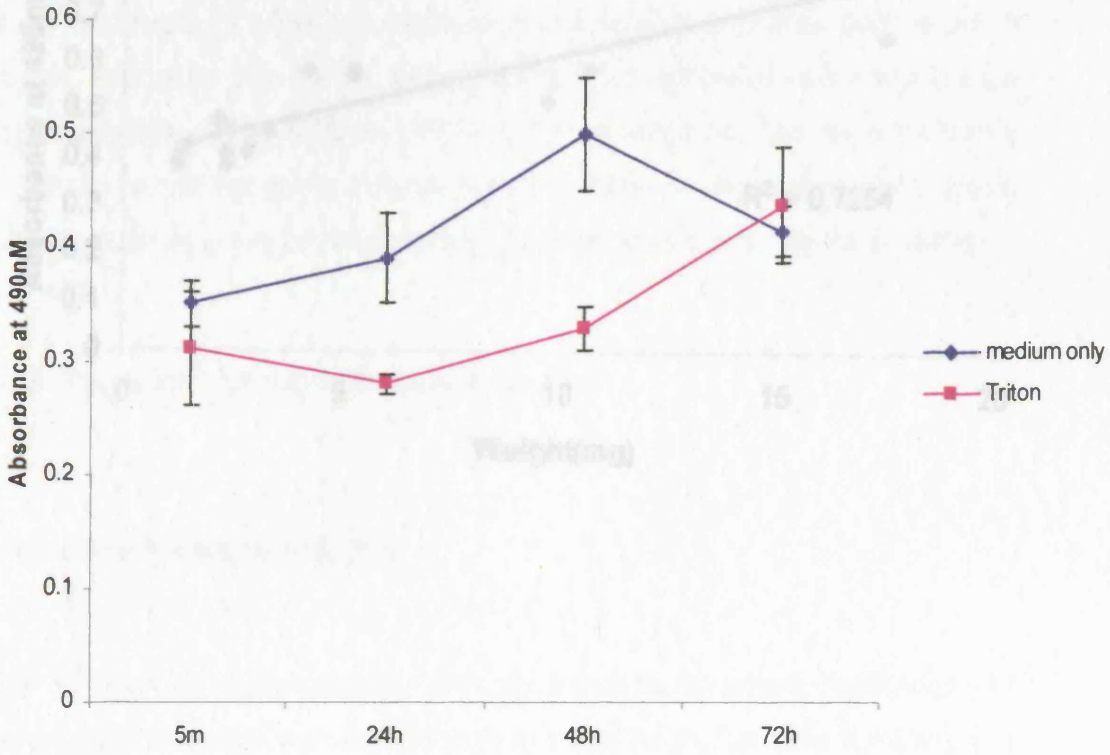
4.5.1. Results of MTS assay

Fig 23 demonstrates the mean absorbances of six experiments. There was a steady increase in MTS up to 48 hours followed by a sharp decline. Triton-X 100 was used as a negative control. By 48 hours of incubation, there appeared to be a drop in MTS and hence a reduced cell proliferation. Using the MTS proliferation assay, therefore, it appears that the explant culture system is viable up to 48 hours.

4.5.2. Results of differing explant weight on MTS absorbance

The MTS assay was performed on 20 explants of differing weights. The results demonstrate a good linear correlation (Spearman Rank, R^2=0.7, p=0.0001) between explant weight and increasing MTS absorbance (Fig 24). For the purposes of future experiments, it was decided to ensure that all explants were approximately the same size in order to minimise variability of results. 1-2mg was taken to be the most physiologically relevant size since good MTS absorbance values were obtained at this size and anything larger with an increased surface area may make penetration of added substrates difficult and may interfere with results.

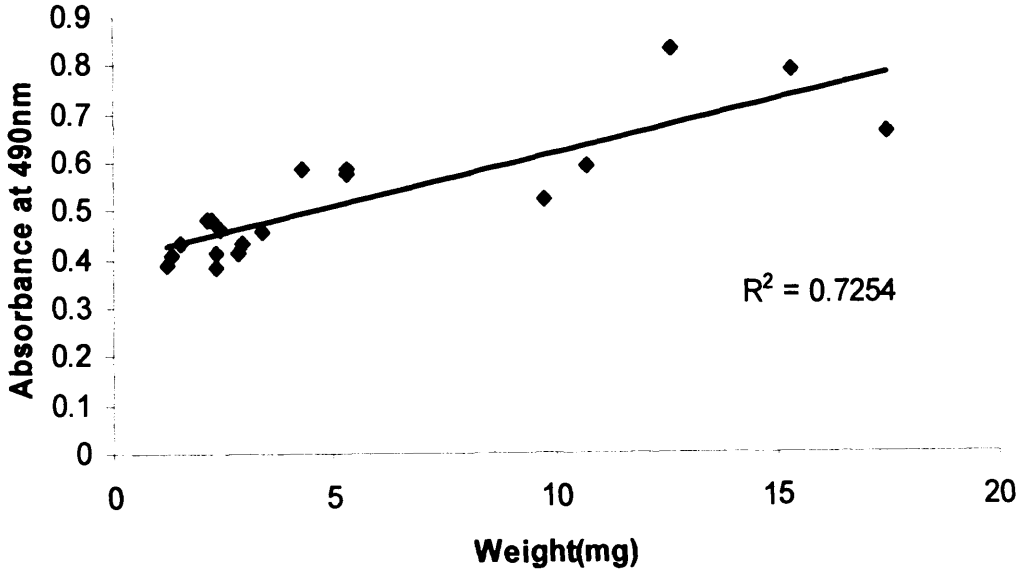
Figure 23. MTS assay of breast tumour explants at 5m, 24hr, 48hr and 72hr. An increase in MTS is seen in the medium only wells compared to negative control, Triton. This confirms the viability of the explants up to 24/48hr, after which point the results appear to be inconsistent. Values are expressed as mean ± SEM.



medium only = medium + tissue explant

Triton = 2% Triton-X 100 in assay medium, negative control.

Figure 24. MTS assay comparing absorbance with differing explant weight. A good linear correlation is seen between explant weights and MTS absorbance.



4.5.3. Results of LDH assay

Figs 25 and 26 show the results of the mean of 6 LDH assays for normal and malignant explants as a guide to their lysed cell content. The results demonstrate a gradual increase in LDH release in the cultured medium of the normal samples after 48 hours. After 72 hours, there appears to be a decline in LDH release suggesting that the culture system is no longer functioning. In the tumours, LDH release is stable for up to 72 hours. Larger amounts of LDH are released from tumour explants compared to normal, probably due to the higher content of epithelial cells and hence proportionately larger release of LDH into the medium. The large increase in LDH from the samples treated with 2% triton (a detergent which lyses cell membranes), the positive control, confirm that the assay is working.

4.5.4. Variability of the LDH assay section

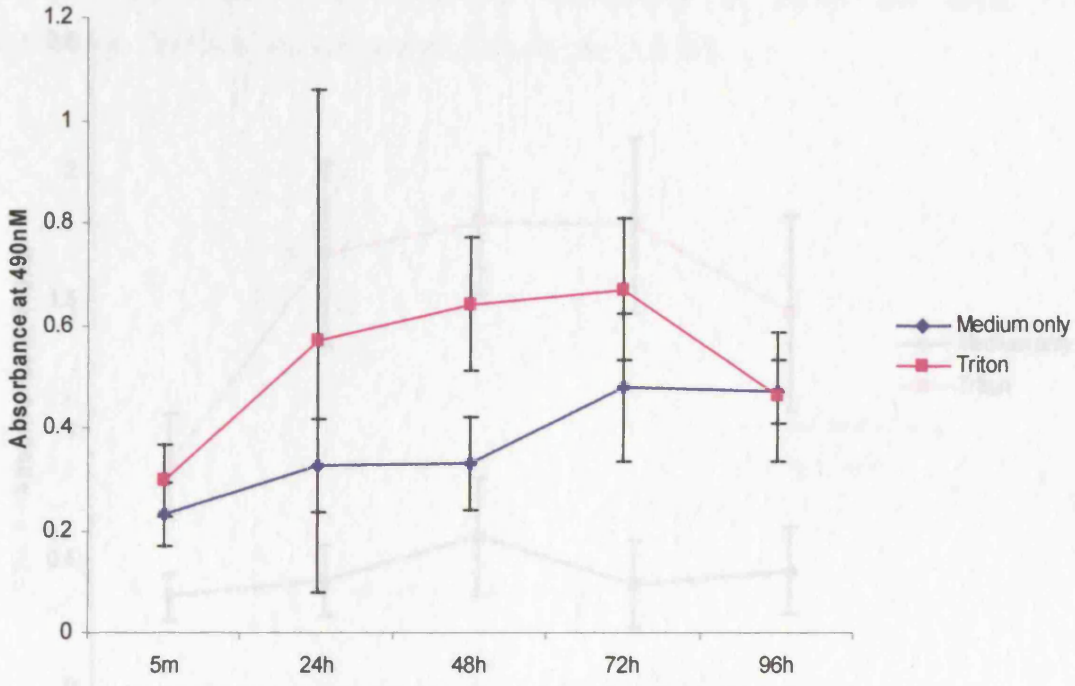
4.5.4.1 Inter-assay variability

Figs 27 and 28 demonstrates the absorbance between duplicates for tumour explants cultured in medium only and triton (positive control). Fig 27 is the mean of 3 experiments at 3hr and Fig 28 is the mean of 4 experiments at 48hr with an increase in LDH seen at this time frame. Good concordance is seen between the samples. The mean co-efficient of variation was 6.6%.

4.5.4.2 Intra-assay variability

Table 11 demonstrates the intra-assay variability for the LDH assay at 5min and 4hr. The mean co-efficient of variation was 3.8%.

Figure 25. LDH assay of normal breast tissue explants (n=6) at 5min, 24hr, 48hr, 72hr and 96hr. Values are expressed as mean ± SEM.



Medium only = medium and tissue explant

Triton = 2% triton-X 100.

Figure 26. LDH assay of breast tumour explants (n=6) at 5m, 4hr, 24hr, 48hr and 72hr. Values are expressed as mean ± SEM.

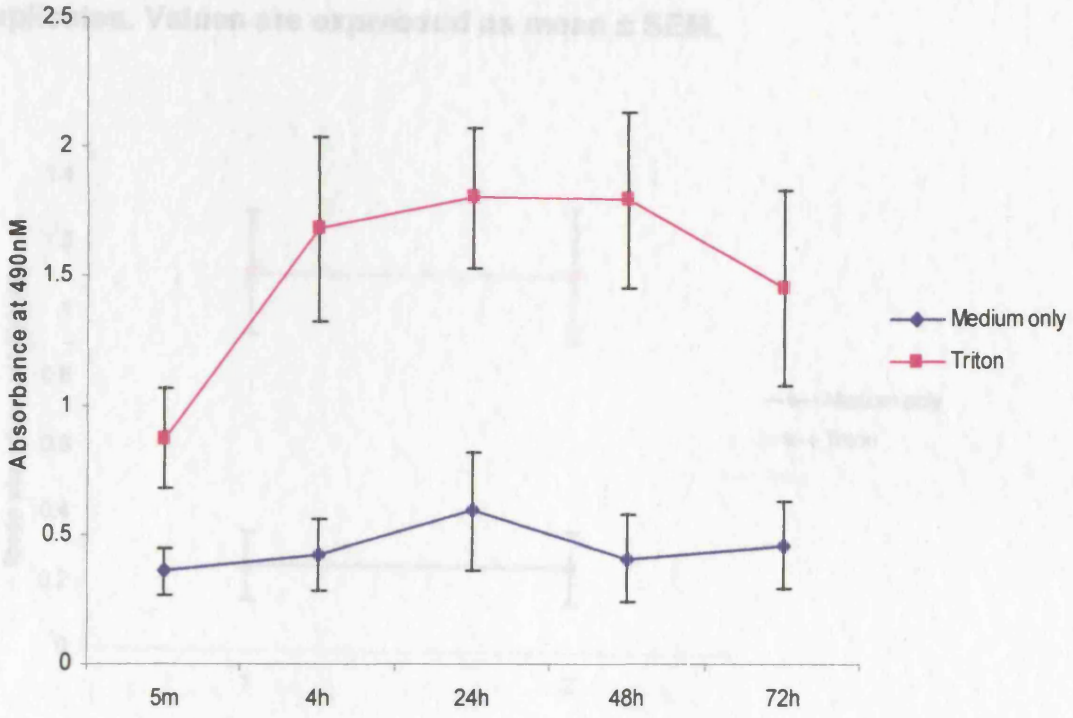


Figure 27. Inter-assay variability of the LDH assay of duplicate tumour explants at 3hr. 1 and 2 on the x axis represent the duplicate samples taken from the same explant (n=3) for explants cultured in medium only and Triton. Good variability is seen for both duplicates. Values are expressed as mean ± SEM.

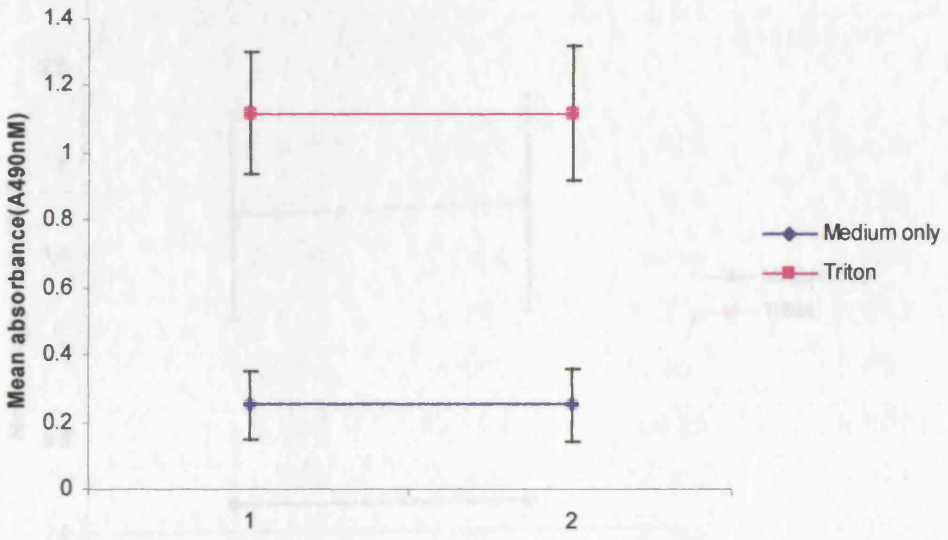


Figure 28. Inter-assay variability of LDH assay of duplicate tumour explants at 48hr (n=4). Good variability is seen in both samples (standard error bars are not shown on medium only duplicates as they are too small, 0.004, 0.005). Values are expressed as mean ± SEM.

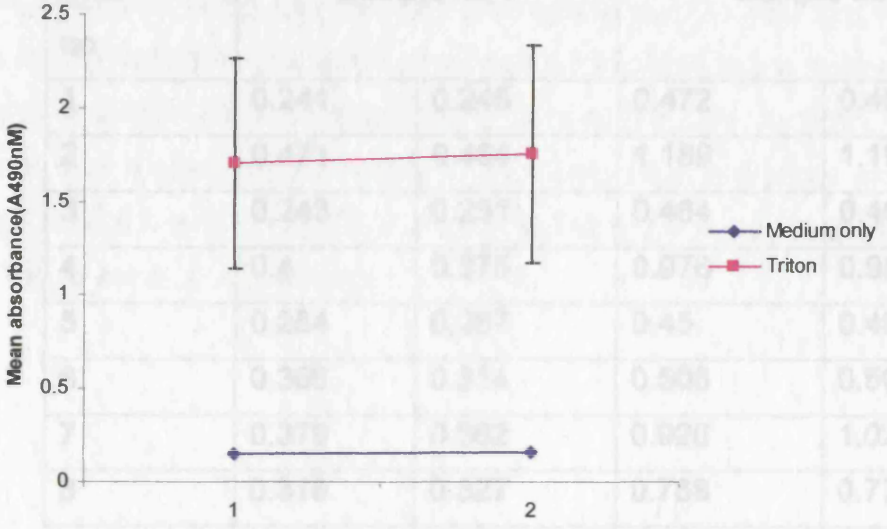


Table 11. Intra-assay variability of LDH assay. Sample 1 and 2 are tumour explants assayed in medium only. Each sample is assayed 12 times in duplicate at 5min and 3hr. The results are measured in absorbance at 490 nM.

	5min		4hr	
Well no	Sample no 1		Sample no 2	
1	0.241	0.245	0.472	0.498
2	0.471	0.464	1.189	1.196
3	0.243	0.251	0.464	0.468
4	0.4	0.375	0.976	0.953
5	0.254	0.267	0.45	0.46
6	0.306	0.314	0.506	0.505
7	0.379	0.362	0.926	1.024
8	0.318	0.327	0.768	0.777
9	0.281	0.286	0.713	0.712
10	0.606	0.573	0.472	0.498
11	0.496	0.511	1.532	1.532
12	0.587	0.526	1.303	1.327

4.5.5. Results of caspase-3 protease assay

4.5.5.1 Results of doxorubicin dose response

Using the caspase 3 colorimetric assay, a dose response for doxorubicin was produced using two differing concentrations; 0.1 mM and 0.3mM in order to investigate the ideal concentration for this culture system. At 4hr there was little effect on apoptosis at 0.1mM (Fig 29). At 24hr, however, there was a significant increase in apoptosis seen in tumour explants at both concentrations (Fig 30). 0.3mM was therefore the dose employed for all future explant experiments.

Figure 29. Caspase-3 protease assay of doxorubicin dose response at 4hr. A minimal effect is seen in breast tumour explants cultured in 0.1mM and 0.3mM doxorubicin. Values are expressed as mean +SEM (n=3).

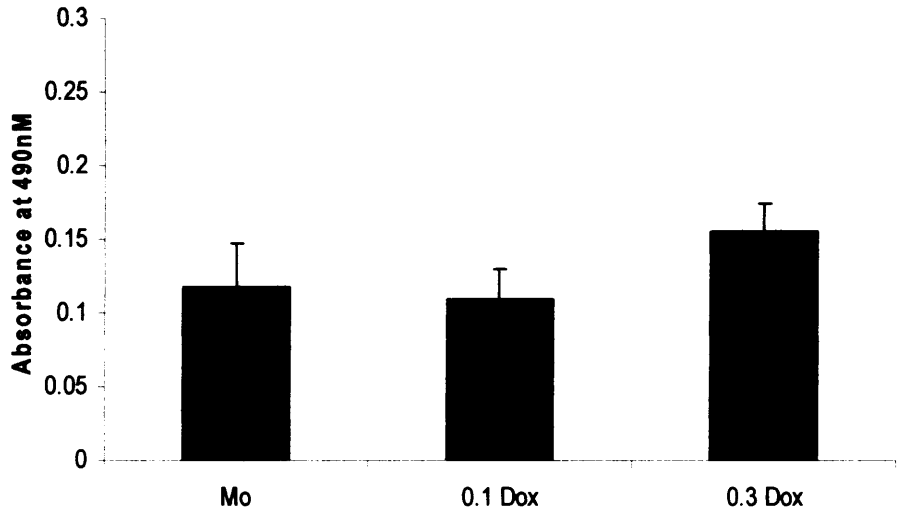
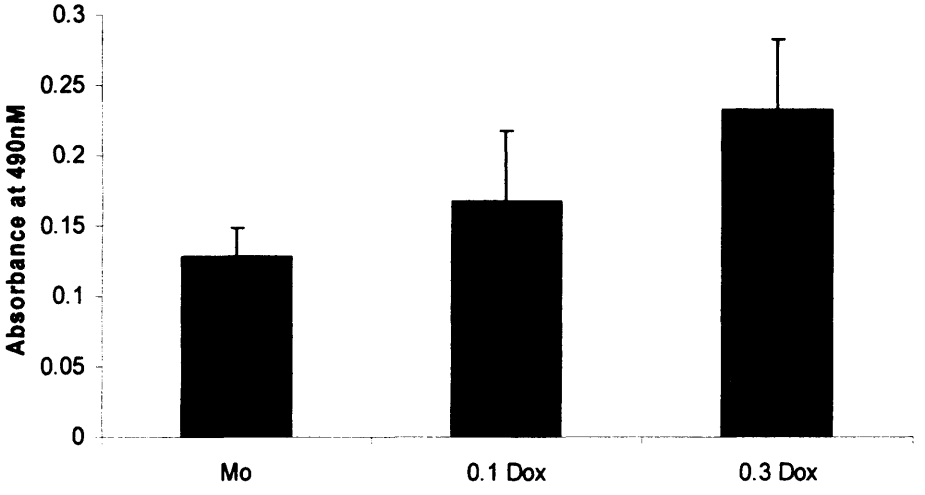


Figure 30. Caspase-3 protease assay of doxorubicin dose response at 24hr. Breast tumour explants showing an increase in caspase activity with 0.3mM doxorubicin. Values are expressed as mean +SEM (n=3).



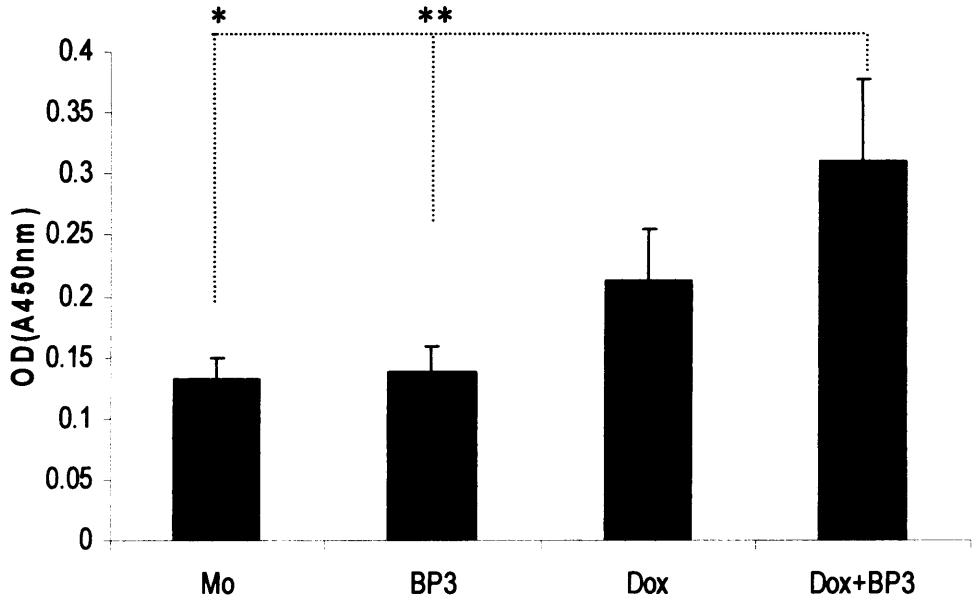
4.5.5.2 Breast tumour explants

Fig 31 demonstrates a small increase in caspase-3 activity in breast tumour explants incubated with IGFBP-3 when compared to the tumour incubated in medium only. This did not, however, reach statistical significance. Similarly, a small increase in apoptosis was seen with doxorubicin, however, this was also not significant. A striking increase in apoptosis was seen, however, with IGFBP-3 in combination with doxorubicin when compared to medium alone ($p=0.004$) and IGFBP-3 alone ($p=0.01$). In breast tumours therefore, IGFBP-3 appears to markedly enhance the apoptotic effects of cytotoxic agent, doxorubicin.

4.5.5.3 Normal breast tissue explants

Fig 32 shows that in normal breast tissue explants, as with tumours, very little apoptosis was seen with IGFBP-3 alone. A significant increase in caspase-3 activity was seen, however, with doxorubicin when compared to medium only ($p=0.02$) and IGFBP-3 alone ($p=0.04$). In contrast to tumours, doxorubicin in combination with IGFBP-3 in normals did not result in a marked increase in apoptosis. Using this explant system divergent effects can therefore be seen in normal and malignant breast tissue.

Figure 31. Caspase-3 protease assay in tumour explants at 24hr. An increase in caspase-3 activity is seen in breast tumour explants (mean of six experiments). Values are expressed as mean + SEM.



Mo = medium only

BP3 = 200ng IGFBP-3

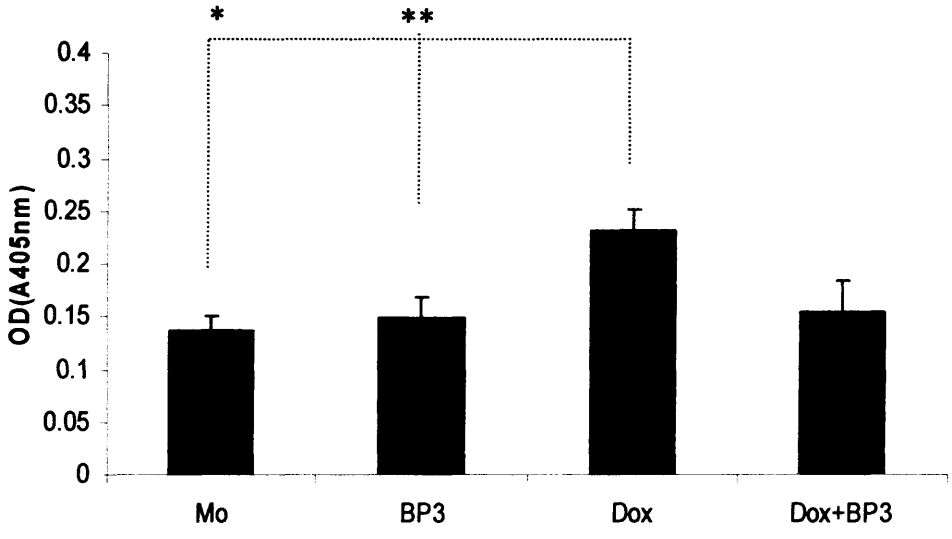
Dox = 0.3mM doxorubicin

Dox+BP3 = 0.3mM doxorubicin + 200ng IGFBP-3

*p=0.004, Mann Whitney U

**p= 0.01, Mann Whitney U

Figure 32. Caspase-3 protease assay in normal explants. An increase in caspase-3 activity is seen in normal tissue explants incubated with doxorubicin at 24hr (mean of 6 experiments + SEM).



*p=0.02, Mann Whitney U

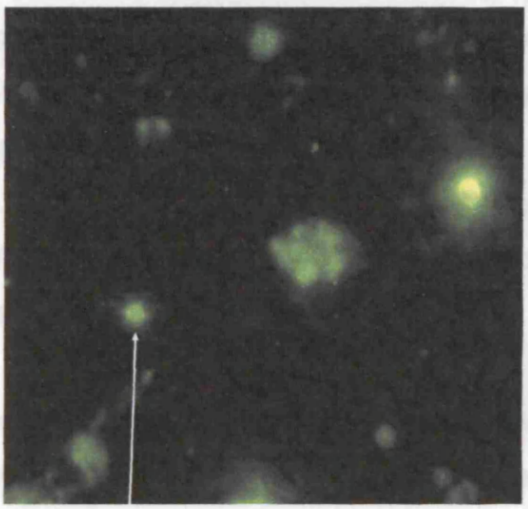
**p=0.04, Mann Whitney U

4.5.6. Results of validation of caspase-3 assay

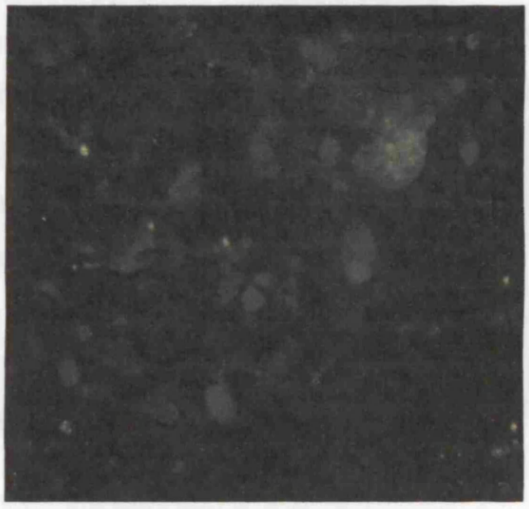
4.5.6.1. Results of immunofluorescence

The results of immunofluroescence were difficult to assess since the reaction was too quick to quantitate apoptosis (Fig 33). Immunofluorescence was therefore abandoned as a quantative validation of the caspase-3 protease assay and only used once in order to compare results using the two techniques.

Figure 33. Immunofluorescence of tumour explants treated with BP-3 and doxorubicin at 24hr. Apoptotic cells were visualised within seconds of staining, however, were not seen 30s later (x100 magnification).



Apoptotic cell seen within seconds



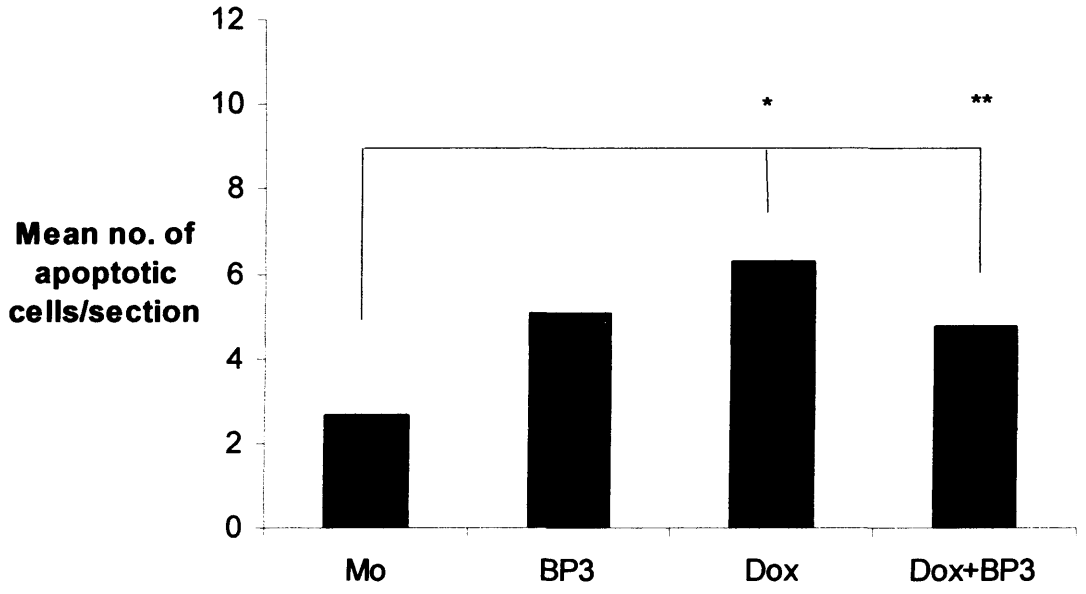
No apoptotic cells seen after 30 seconds

4.5.6.2. Results of anti-caspase-3 immunohistochemistry

An increase in apoptosis was seen in the tumour explants cultured with IGFBP-3, doxorubicin and IGFBP-3 in combination with doxorubicin at 24 hours (Fig 34). The mean number of cells undergoing apoptosis was statistically higher in the tumours cultured with doxorubicin alone and doxorubicin in combination with IGFBP-3, when compared to the tumour cultured in medium alone ($p<0.05$). There was, however, no statistical difference detected in the number of apoptotic cells seen in the tumours that were cultured with IGFBP-3 alone and similarly, the addition of IGFBP-3 to doxorubicin seemed to have no additive effect. Only 2 experiments were performed, however, due to the difficulties encountered in obtained enough tissue for all experiments. The results therefore, reflect a trend and further samples need to be performed to confirm these results.

Several cells per section stained positively with anti-caspase 3 antibody, however, some of these cells appeared to have completely undergone apoptosis whereas others were at different stages in the apoptotic pathway (Fig 35). The final results, therefore, reflect the total number of cells that have completely undergone apoptosis and are undergoing the process of apoptosis.

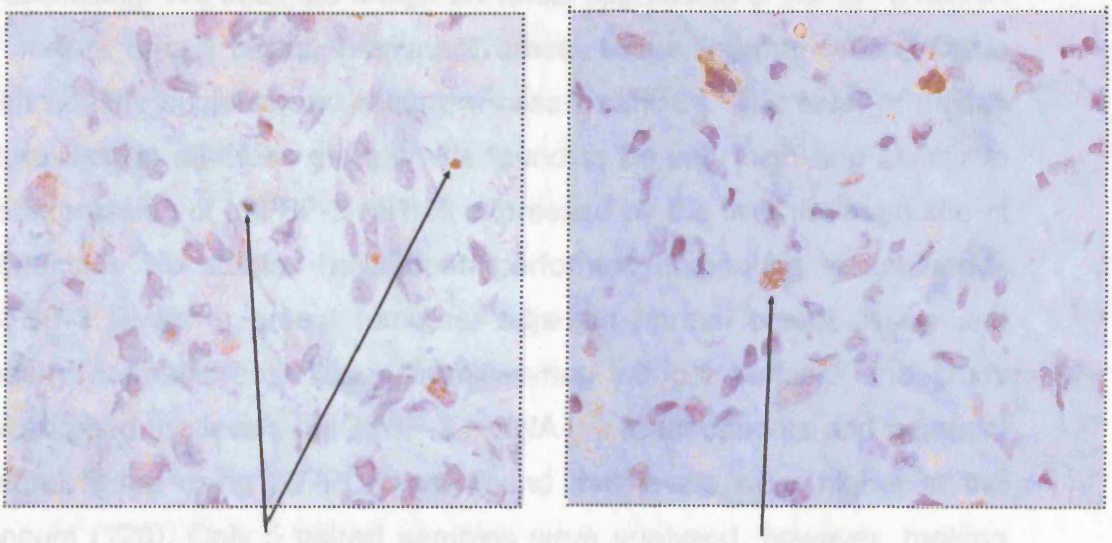
Figure 34. Anti-capase 3 immunohistochemistry of tumour explants. Mean number of apoptotic cells/section of tumour explant cultured at 24hr (mean of 2 experiments, approximately 10 sections/explant, magnification x200).



* p=0.04

** p=0.05

Figure 35. Positive anti-caspase 3 staining in tumour explants. Some cells have completely undergone apoptosis (apoptotic bodies) and others are undergoing the process.



5. Discussion

5.1. IGFBP-3 expression in breast tissue

This study was performed to confirm the expression of IGFBP-3 in human breast tissue. Previous *in vitro* studies have shown that IGFBP-3 regulates growth of breast cancer cells (11;34;120), however, *in vivo* data is conflicting. We detected a high universal expression of IGFBP-3 mRNA in normal breast tissue, malignant breast tissue, normal breast tissue from healthy subjects and recurrent breast cancers. The level of mRNA expression in all these groups was found to be very high and similar to the expression of IGFBP-3 mRNA expressed by the liver, its main site of production. No studies have been performed comparing simultaneous IGFBP-3 levels in breast tumours, adjacent normal breast tissue and healthy normal breast tissue from women without cancer. One study investigated the levels of IGFBP-3 mRNA in breast cancers and adjacent normal tissue using RT-PCR and found that levels were higher in the cancers (120). Only 5 paired samples were analysed, however, making statistical significance difficult to assess.

The over expression of IGFBP-3 by aggressive breast cancer cells and samples initially appears somewhat of a paradox since IGFBP-3 is generally considered to be a growth inhibitor. It is possible, therefore, that in fact the over expression of IGFBP-3 is from the nearby normal tissue and stroma in response to the aggressiveness of the tumour. This may, therefore, reflect a local response of the body to try to limit the development of the breast tumour. There was, however, no statistical difference seen between the IGFBP-3 expression of normal and malignant breast tissue or any of the other types of breast tissue. If the local production of IGFBP-3 in normal breast tissue is affected by the presence of a breast tumour, we might therefore expect to see a

difference in IGFBP-3 levels between normal breast tissue from healthy women and those with breast cancer. This finding, however, was not reflected in the mRNA data.

5.1.1 Limitations of RT-PCR

Real-time RT-PCR is now widely used for characterising or confirming gene expression patterns and comparing mRNA levels in different sample populations. Recent improvements in internal standards, references for normalising data and new mathematical models to analyse data have improved its use as a possible clinical diagnostic tool. Doubts still exist, however, about how quantitative and informative real-time RT-PCR is. A wide variety of protocols exist amongst research laboratories, similarly there is no universally accepted validation of normalising data thereby leading to difficulties in reproducing data. Recent improvements have led to the development of standardised templates (257) which allows comparison between experiments and use of internal standards to reduce variation in template starting amounts and operator loading error (258).

One of the major problems with this study is the *in vivo* RNA extraction from whole tissue samples. The heterogeniety of cell types within the sample leads to an averaging out of the expression in different cell types and perhaps the expression profile of a specific cell type may be masked or even lost. Previous studies have shown significant differences in gene expression profiles of microdissected and bulk tissue samples (259;260). This may be particularly problematic when comparing normal and malignant tissue since normal tissue directly adjacent to a tumour may be phenotypically normal when infact it displays an altered gene expression due to its proximity to the tumour (261). In order to overcome this problem, we obtained normal breast as far away as possible from the main tumour site (average of 10cm) which was then confirmed as microscopically normal breast tissue by the pathology department.

Laser capture microdissection (LCD) is a novel technique that allows the extraction of a pure subpopulation of cells from heterogenous in vivo tissue samples (262). A brief laser pulse is directed at cells within a tissue section on a glass slide and individual cells may be selected and gene expression may then be determined at an RNA and protein level. The main drawback of this technique is that a very small sample is yielded making RNA isolation difficult and good quality RNA is essential for successful RT-PCR. The technique is also classically performed using archival formalin-fixed paraffin-embedded tissue specimens and the RNA yielded from these slides may have undergone extensive degradation and also the use of fixatives may well alter the results (263;264).New commercial kits have been developed in order to tackle these problems, however, and so future use of this technique for this study may well improve the validity of these results.

5.2. IGFBP-3 protein expression

IGFBP-3 protein was detected in 96% of all samples, thereby corroborating the high level of expression in all samples detected using RT-PCR. Unfortunately, however, no statistically significant correlation was seen between protein expression and mRNA of IGFBP-3 in these samples ($R= -0.2$, $p=0.17$). This may be due to sample size, however, we should expect to observe a trend with the sample size studied. Accurate quantification of mRNA levels provides no information on transcription levels or mRNA stability. Also, mRNA data may not reflect the levels of protein produced by the cell as regulation occurs at the post-transcriptional stage (265). RT-PCR data may therefore be very uninformative about protein activity. Previous studies have demonstrated a strong correlation between IGFBP-3 mRNA and IGFBP-3 detected by immunoradiometric assay (IRMA) but no statistically significant correlation was seen between IGFBP-3 mRNA and IGFBP-3 detected by ligand blot or immunoblot (153). Ligand blot and immunoblot are,

however, only semi-quantative whereas IRMA is more sensitive and precise suggesting that IGFBP-3 levels may be directly related to their mRNA and therefore transcriptionally upregulated in breast cancers. This difference in findings may be accounted for by the different techniques used to measure IGFBP-3 mRNA and protein.

When analysing the pattern of expression of IGFBP-3 protein, there is a significant increase in stromal staining. This is a novel finding and has not previously been reported in breast cancer. This is an interesting finding given the increased epithelial content of breast tumours, where we would perhaps expect to find an increase in epithelial staining compared to the normal tissue. In colon cancer, however, IGFBP-3 protein was detected predominantly in the epithelium with less BP-3 protein being expressed in the poorly differentiated adenocarcinomas (39). It was suggested in this case that BP-3 causes apoptosis via a p53-dependant mechanism and so loss of p53 function may lead to a down regulation of BP-3 protein potentially leading to aberrant cell survival. However, one study has suggested that the DNA sequence of BP-3 is altered in the normal and malignant tissue of patients with colon cancer, thereby the patient may have been predisposed to developing the cancer (266). Further studies would be required to analyse protein expression in the breast tissue from non-cancer patients for comparison.

5.3. BP-3 expression and clinico-pathological features

We detected a significant correlation between breast tumour tissue sections that stained negative/weakly positive for BP-3 and lymph node negativity ($p=0.05$). This again is in contrast to colon cancer where less BP-3 protein is seen with more aggressive cancers (267).It is further evidence that breast tumours may behave differently to colon cancer and supports a potential role for IGFBP-3 in the development of invasive breast disease. It further adds to the epidemiological evidence from Renehan *et al* that high serum levels of BP-3 are associated with an

increased risk of breast cancer (98). It is possible that the high levels of BP-3 produced by the surrounding stroma have anti-apoptotic effects on adjacent epithelium thereby aiding the development of invasive cancer or infact, the tumour may have become resistant to the pro-apoptotic effects of BP-3 from the stroma.

One previous study measured IGFBP-3 using ELISA in 195 node negative tumours (268). Their findings agreed with our data in that all samples expressed high levels of IGFBP-3. However, they did not correlate BP-3 levels in this group with patients who were lymph node positive so it is difficult to determine whether their findings are consistent with ours. IGFBP-3 levels were, however, inversely correlated with age and ER/PgR status and found to be higher in tumours greater than 2 cm. Our data did not demonstrate any such association with hormone status or tumour size. This may be as a result of the limitations of the sample collection. The majority of samples were taken from post-menopausal women with tumours that were 2cm or greater since it was technically difficult to obtain samples from smaller specimens without compromising the margins for analysis. Also, approximately 85% of samples were ER positive reflecting the usual hormonal status in all breast tumours, however, resulting in a small sample size of ER negative tumours in this study. Our data, however, did reflect a general trend towards high levels of expression of IGFBP-3 mRNA in larger tumour over 2cm (75% of samples expressed > 1.0×10^8 copy numbers) and ER negative tumours (78% of samples expressed > 1.0×10^8 copy numbers). Therefore, with a greater variety of samples, we may be able to demonstrate similar findings. No other association was found between IGFBP-3 levels and the following clinico-patholgical variables; tumour grade, histological type and presence of vascular invasion.

Another study measured IGFBP-3 protein levels in 47 cancers using western ligand blot with $^{[125I]}$ IGF-I as a label (120). They found that IGFBP-3 levels were significantly higher in ER negative tumours, which are known to predict for a poorer outcome. Similarly Yu *et al* measured

IGFBP-3 levels in tumour tissue cytosols from 169 breast cancers. They found that IGFBP-3 levels were inversely correlated with ER status and positively correlated with S phase fraction, known to be a powerful predictor of prognosis in node negative cancers (269). In another study by Figueroa *et al*, IGFBP-3 was measured by western ligand blot (WLB) and immunoblot analysis in 40 breast cancers. IGFBP-3 mRNA was directly quantified by scanning the gel with a radio-analytical system and results showed that low levels of IGFBP-3 were expressed but were increased in ER negative tumours (270).

Shao *et al* found similar results using in situ hybridization and image cytometry (121). In another study of 40 breast tumours by Rocha *et al*, IGFBP-3 levels were measured by ligand blot analysis, immunoblot analysis, immunoradiometric assay (IRMA), and ribonuclease protection assay. High levels of IGFBP-3 were found to be associated with poor prognostic features such as ER/PgR negativity, high S phase and aneuploidy (153). Shao *et al* felt that the higher expression of IGFBP-3 by ER negative tumours may play a role in the rapid proliferation and poor prognosis of these tumours.

Two studies have measured IGFBP-3 mRNA. One used in situ hybridisation and image cytometry in 20 breast cancers and found that there was some overlap between the ER positive and negative samples (121). The other study performed RNase protection and video densitometry on 40 cancers and found no correlation with ER status.

For analysis purposes, our IGFBP-3 mRNA data was divided in two groups; tumours expressing high levels of IGFBP-3 mRNA ($> 1.0 \times 10^8$ copy numbers) and low levels of IGFBP-3 ($<1.0 \times 10^8$ copy numbers). In the group expressing high levels, a significant increase in the presence of high grade ductal carcinoma in-situ (HG DCIS) was seen when compared to the group expressing lower levels. DCIS is the precursor to the development of invasive breast cancer and can predict for a more aggressive clinical outcome. This finding has not previously been

reported and is further evidence for a role of BP-3 in the development of invasive breast cancer. These findings therefore suggest that high levels of IGFBP-3 may be involved early on in the process of breast tumourigenesis and may predict for a more aggressive outcome. One similar study found a positive correlation between IGF-I and presence of DCIS (102), however, nobody has looked at the association with BP-3.

Previous studies, therefore, demonstrate conflicting evidence for the association between IGFBP-3 levels and poor predictive markers for breast cancer. We have clarified the inverse correlation of IGFBP-3 expression with lymph node positivity and similarly the positive correlation with DCIS. Both of these factors predict for a more aggressive outlook in breast cancer.

5.4. IGFBP-3 and survival

By the end of the study, four deaths had occurred (mean follow up 20 months) and all four were from the group expressing high levels of IGFBP-3. Due to small sample size this did not, however, reach statistical significance.

One study measuring IGFBP-2, 3, 4 and 5 protein in 238 samples by immunoblotting, found that none of the IGFBPs were independent prognostic indicators of disease free survival using univariate analysis (271). Other clinical studies have also found no significant correlation between IGFBP-3 levels and disease recurrence and survival (268;272). Similarly, no relationship was found between serum IGF-I levels and survival in a study looking at advanced breast cancer (273).

5.5. BP-3 expression and tumours associated genes

VEGF is known to be up-regulated early in the development of several cancers, particularly colon (274). It is known to play an important role in tumour angiogenesis (275) and *in vitro* studies have shown that IGF-I up-regulates VEGF transcription (276). No previous studies have reported on any association between BP-3 and VEGF. We detected no significant correlation between VEGF and IGFBP-3 mRNA.

COX-2 is known to play an important role in tumorigenesis. Considerable evidence suggests that use of COX-2 inhibitors is associated with a reduced incidence in gastro-intestinal malignancy probably via their inhibition of cyclo-oxygenase (COX) activity and subsequent decreased production of prostaglandins (277). Studies of NSAID use in breast cancer are, however, inconsistent. A recent meta-analysis of NSAID use in breast cancer quoted an 18% reduction in breast cancer risk (278). COX-2 in colon cancer has also been reported to be directly involved in tumour angiogenesis and therefore may be associated with prognosis (279;280). Interestingly, we detected a significant increase in COX-2 mRNA in normal breast tissue adjacent to breast cancer when compared to normal tissue from healthy patients. No correlation was found, however, between IGFBP-3 and COX-2 mRNA. This suggests that COX-2 may play a role in breast tumourugenesis, however, perhaps not via induction of IGFBP-3.

5.6. BP-3 expression and vitamin D

Vitamin D is known to inhibit proliferation of breast cancer cells *in vitro* and *in vivo* (197) and has been shown to block the mitogenic effects of IGF-I (281-283). The mechanism by which this occurs is unclear. It may be due to down regulation of the IGF-IR (282), an up regulation of

inhibitory IGFBPs or perhaps a modulation of down stream effectors of IGF-I signalling. An *in vitro* study performed by Colston *et al* demonstrated an increased expression of IGFBP-3 in MCF-7 and Hs578t breast cancer cells treated with vitamin D which were not stimulated by IGF-I (184).

The striking observation from this data is the reduced expression of 1αOHase mRNA in healthy tissue from patients with breast cancer compared to that from patients with benign disease. This expression pattern is similar to the one we have previously reported in the colon (284;285). There are three possible explanations for this finding. First is that it is merely a reflection of age as the samples of normal breast from women without cancer were from younger women than those with cancer. However, there was no correlation between 1αOHase mRNA levels and age in either the normal controls or the adjacent normal breast tissue from cancers. Also, there was no statistical difference in mRNA levels in the NN samples between younger (<40 years) and older (>40 years) patients. Second, the down-regulation in adjacent healthy tissue may reflect a field effect of the tumour. Our results indicate that this is not the case with 1αOHase mRNA levels approximately constant between normal breast tissue immediately adjacent and up to 10 cm away from the cancer. A similar result was obtained in normal colonic tissue from patients with colon cancer (unpublished observations). Third, there is a general mechanism of dysregulation of the vitamin D axis in breast tissue of women who subsequently develop breast cancer. This could result in a predisposition to tumour development given the anti-proliferative and pro-apoptotic effects of active vitamin D. Previous work has implicated aberrant promotor methylation as an important mechanism for the development of both breast and colon cancers and hypermethylation of the 1αOHase gene promotor has recently been reported in more than 40% of primary breast cancers. Our data imply that such hypermethylation may be present in normal tissue and account for the observed downregulation. If present, it would suggest that disruption of

this hypermethylation might offer novel chemopreventive approach to breast cancer.

Interestingly, we observed a positive correlation between IGFBP-3 and 1 alpha hydroxylase mRNA levels (R=0.4, p= 0.01). 1 alpha hydroxylase is the enzyme responsible for the activation of vitamin D which is known to have anti-proliferative and pro-apoptotic effects on breast cancer cells (184). It has been shown to be expressed at high levels by the kidney and similarly, we have demonstrated its expression in breast cancers. Previous cell line work has suggested that vitamin D may exert its anti-proliferative effects by up-regulation of IGFBP-3 (184). Our results support this hypothesis. No other correlation was detected between IGFBP-3 and other members of the vitamin D axis; vitamin D receptor (VDR) and 24 hydroxylase, the enzyme responsible for breaking down active vitamin D.

We also confirmed the up regulation of VDR mRNA in breast tumours as has previously been reported (286;287). A previous study suggested that vitamin D up regulates IGFBP-3 mRNA (182) and so this positive correlation therefore agrees with previous data. This effect may occur via the induction of TGF-β which is known to be a potent modulator of IGFBP-3. Other studies, however, have found an enhanced expression of IGFBP-5 protein with vitamin D induced cell growth inhibition (288). Further studies need to be performed to further elucidate whether vitamin D can influence IGFBP expression or infact, directly modulate down stream effectors of IGF-I signalling. It may be possible to demonstrate such effects using our primary explant system.

We did not observe any association between either 1αOHase or VDR mRNA levels and oestrogen receptor status, histological grade of tumour, presence of vascular invasion or lymph node metastases. In contrast, in breast cancer cell lines expression of 1αOHase has been reported to be higher in well differentiated, estrogen receptor (ER) positive cells.

In conclusion, these early data confirm the importance of the vitamin D axis in breast cancer and suggest that assessment of 1αOHase mRNA levels in women may be useful in assessing risk of future breast cancer development.

5.7. Serum IGF-I and IGFBP-3 levels

IGF-I levels were significantly lower in patients taking tamoxifen, however, no effect was seen on serum IGFBP-3 levels. The IGF-I: BP-3 ratio was also found to be significantly higher in women with breast cancer prior to surgery, thereby agreeing with previous epidemiological studies. Tamoxifen has been shown to significantly reduce serum levels of IGF-I (65;168;289-294) but to have no effect on IGFBP-3 (177). One study, however, found that patients taking Tamoxifen had decreased IGFBP-3 protease activity thereby implying that an increased IGF-I may be available in the circulation (65). Circulating levels of IGF-I and IGFBP-3 are, however, strongly affected by nutritional status (295;296) and the catabolic stress that accompanies surgery (62;297). In this study no fall in IGF-I was observed in the group not taking Tamoxifen post-operatively, however, at 6 months we would expect that IGF-I levels may have returned to normal. Similarly, the stress of surgery appeared to have no effect on the serum concentrations of IGFBP-3, possibly due to a decrease in protease activity.

The mechanism by which Tamoxifen alters the IGF-I axis has not been fully elucidated. It is thought that the oestrogen receptor blockade by Tamoxifen interferes with the production of growth hormone from the pituitary, thereby lowering the amount of IGF-I produced by the liver and released into the circulation. One study has also suggested that Tamoxifen may work via a pituitary –independent action of reducing IGF-I in the peripheral tissues usually favoured by breast cancer metastases (298). It also has direct anti-oestrogen effects on breast cancer cells and

may well influence the amount of IGF-I and IGFBP-3 produced by the cells themselves (168;176).

Interestingly, one study has shown that breast cancers with acquired anti-oestrogen resistance continue to express ER of normal size and DNA-binding ability and suggest that the failure of anti-oestrogens to arrest tumor growth may be a result of IGF-I mediated signal transduction pathways and its interactions with ER signalling (299). Our own mRNA data has shown a significant down regulation of IGF-I in recurrent breast tumours when compared to primary tumours ($p=0.0001$) with no difference seen in BP-3 levels. This down regulation may be secondary to the effects of Tamoxifen, or may suggest that IGF-I is in fact not a feature in the development of tamoxifen resistance. Further studies in this area are required.

5.8. Primary breast explant culture

Most research has focused on the role of IGFBP-3 as a modulator of IGF-I action. There is, however, increasing interest into its additional IGF-I independent, pro-apoptotic effects. Previous studies investigating the biological actions of BP-3 have mostly involved *in vitro* cell culture work (300) where results may not necessarily reflect physiological *in vivo* condition or have used insensitive techniques such as northern blotting (120).

In order to clarify the effects of BP-3 on breast cancer, we developed a novel, physiologically relevant, explant system to provide us with more clinically relevant information than cell line work can provide. The main difference in methodology involved the use of breast tissue which retains its architecture, thereby avoiding the use of cancer cell lines which have previously undergone many mutations and may not be physiologically relevant. There were, however, a number of potential areas for error in experimental technique. One variability factor was the time taken to

transfer the tissue from the patient into the media. In order to standardise this and mimic *in vivo* conditions, all samples were collected immediately after surgical excision and placed into media warmed to 37°C. 45 min was allowed for tissue transfer. Similarly, another variability factor in comparing the samples is a difference in weight and composition of the tissue. Normal breast tissue particularly, consists of varying amounts of fat and fibrous tissue which may influence the weight. To standardize this factor, all samples were cut into sizes weighing approximately 10 – 20mg. Every effort was made with normal breast tissue to excise all fatty tissue away first so that only glandular breast tissue was used in the experiments. Similarly we found a good correlation between absorbance and weight up to 20mg in weight, thereby further standardizing this technique. Ideally, in addition to standardizing for weight, adjusting for protein or DNA content would improve the standardization of the results and should be considered for future experiments.

The LDH and MTS assays and standardization of absorbances allowed us to validate our novel culture technique. Common methods for determining cell line viability depend upon membrane integrity (e.g. trypan blue exclusion), or incorporation of nucleotides during cell proliferation (e.g. BrdU or 3H-thymidine). These methods are limited by the impracticality of processing large numbers of samples, the requirement for handling hazardous materials and the lack of penetration into solid samples. The MTS Assay is a rapid method of assessing cell viability and is used to measure changes in cell viability or proliferation. In actively metabolising cells, an increase in MTS conversion is spectrophotometrically quantified. Comparison of this value to an untreated control provides an increase in cellular metabolic activity. Conversely, in cells that are undergoing apoptosis or necrosis, MTS reduction decreases, reflecting the loss of cell viability.

Similarly for assessment of cell death we used the LDH assay. Other widely used methods include uptake or exclusion of vital dyes such as

trypan blue, eosin Y, nigrosine, propidium iodide or ethidium bromide (301). Dead and viable cells are discriminated by differential staining and counted using a light or fluorescence microscope. These methods are time consuming and do not account for dead cells which may have lysed. The actual rate of cell death, therefore, may actually be underestimated in the long term. Limitations of this assay included a large variation in results between normal and tumour explants which may be as a result of the higher epithelial/stromal ratio seen in tumours. It may be, therefore, be difficult to directly compare the results from normal and tumour explants.

We found that IGFBP-3 caused little apoptosis when used alone on normal and malignant breast explants. Several studies have suggested that IGFBP-3 exerts inhibitory, IGF-I independent effects on cell function. Rajah *et al* (18) demonstrated that IGFBP-3 directly induced apoptosis on breast cancer cells, however, in this study we did not observe such effects. Our results agree with Holly *et al* (107) who used Hs578T cells. The reason for differing results in cell line studies may be attributable to a difference in cell culture conditions. Our results using fresh tissue explants, however, do agree with previous work (107). Greater number samples, however, would be necessary to make better overall judgements from the data obtained.

We observed a marked enhancement of apoptosis when IGFBP-3 was used in conjunction with known cytotoxic agent, doxorubicin. Our data is consistent with other studies where IGFBP-3 induced apoptosis has been accentuated by various types of cell stress, such as irradiation, ceramide analogues and cytotoxic agents (36;39;41;114). The mechanism by which IGFBP-3 accentuates apoptosis in these circumstances is unclear. Holly *et al* suggested that IGFBP-3 may exert its IGF-I independent effects through direct or indirect interactions with integrin receptors (107). The integrin receptors have a well characterised role in maintaining cell survival (302) as well as mediating cell attachment. They found that focal adhesion kinase (FAK), a key mediator of integrin signalling, is dephosphorylated in the presence of IGFBP-3. The physiological

significance of this finding remains to be determined, however, it is possible that the apoptotic effects of IGFBP-3 may occur by modulation of survival signals transduced via integrin receptors.

Tumour suppressor gene, p53, is a critical regulator of the transcription of many cellular genes that are involved in mediating cell cycle arrest and apoptosis (303).Many studies have demonstrated the importance of p53 as a tumour suppressor, however, little is known about the effect of loss p53 on human cells in the context of ECM regulatory signals. Interactions between breast epithelial cells and ECM play an important role in maintaining normal tissue homeostasis and are likely to be disrupted in breast tumorigenesis. IGFBP-3 was identified as a p53-inducible gene and a mediator of p53-dependent apoptosis in EB1 colon cancer cells in response to cellular stress .Similarly, another study reported that IGFBP-3 potentiation of apoptosis after ionizing radiation in colon cancer cells was dependent on functional p53 (39). Loss of IGFBP-3 function would therefore lead to loss of sensitivity to growth inhibition during cellular stress i.e irradiation and hence resulting in an increase in cells which fail to express functional IGFBP-3 protein. Williams *et al* therefore concluded in this study that IGFBP-3 secretion may contribute to colorectal tumorigenesis because loss of IGFBP-3 response prior to acquisition of a p53 mutation may result in overgrowth of an aberrant cell population.

Interestingly, we have demonstrated differential effects on apoptosis in normal and malignant breast tissue. Apoptosis induced by doxorubicin was similar in both normal and malignant breast tissue, however, the addition of IGFBP-3 markedly enhanced apoptosis in tumours, whereas the reverse effect was seen in normal breast tissue. In these samples, far less apoptosis was observed suggesting that IGFBP-3 may have a protective effect on normal breast tissue. This is a novel finding and is in accordance with previous work by McCaig *et al* (107) who used ceramide to induce apoptosis on normal breast cell line, MCF-10A. The differential effects may also be explained by the difference in epithelial and stromal content of the tissue types. In tumours we would expect to find significantly more epithelial cells and less stromal tissue and in normal tissue, it would predominantly be stroma. IGF-I is known to be produced

by stromal tissue and we have shown that BP-3 is also expressed mainly by stroma and so this may account for the differences in apoptosis.

We corroborated the results by counting apoptotic cells on immunohistochemical slides. It is possible also that this method of detecting apoptotic cells was not sensitive enough to detect the differences. Some problems encountered involved identifying the cells that were actually undergoing apoptosis as some appeared to being undergoing apoptosis whilst others had completed the process. It was decided to include cells at all stages in the process for the final calculations. More samples would be needed, however, to draw further conclusions.

A study by Holly *et al* (107) also demonstrated a differential effect of IGFBP-3 with tumour cells grown on plastic and fibronectin. On plastic, ceramide induced apoptosis was enhanced by IGFBP-3, however, reduced apoptosis was observed with fibronectin. This suggests that the extra-cellular matrix (ECM) may play a role in the differential effects of IGFBP-3. The ECM was obviously intact in our experimental technique, therefore it is difficult to directly compare the two studies. In addition, the use of serum free medium allows us to believe that the different effects observed, are due to BP-3 itself and not any growth factors present in the medium.

The viability of the cultured tissue was crucial to the validity of the explant system. Two different methods were used to assess viability; MTS and LDH assays which measured cellular activity and lysis. These assays were chosen as they are simple and sensitive techniques which measure two different biological end-points. There were, however, a number of potential areas for error in experimental technique for both assays. Firstly, care was taken to minimise pipetting error when adding the dye in each well. Secondly, care was taken during pipetting to avoid generating bubbles within each well as this interferes with the fluorescence measurement. Both assays were light sensitive, therefore all incubation

took place in the dark. For the MTS assay, incubation time after adding the dye was maintained at 4 hours for all samples in order to standardize results and allow the dye adequate time to diffuse into the tissue. The negative control for both assays was a tissue explant cultured in medium only. For the MTS assay, an additional negative control was used. A tissue explant was frozen in liquid nitrogen and then incubated, as before, in medium only. Absorbance values for this frozen negative control were lower than absorbances for the non-frozen negative control, thereby further supporting the assumption of viability of tissue explants with high MTS absorbances up to 48 hours. Similarly, with the LDH assay, a positive control of 2% Triton was used which causes complete membrane disruption. Absorbance values from these samples were much higher than the experimental tissue exlants, thereby further supporting assay viability. The 6 normal explants appeared to maintain steady LDH release and viability up to 72 hours after which it tails off. The 6 tumour explants, however, demonstrated on overall increased amount of LDH at each time frame up to 24 hours, tailing off after this and thereby suggesting decreased viability. There was some discrepancy in the results of tissue viability recorded by MTS and LDH. It may be, therefore, these two assays cannot be compared directly. Other media absorbances could be compared to this 100% membrane-disrupted value.

The differential effects of IGFBP-3 demonstrated in the primary explant system suggest a protective role for IGFBP-3 in normal breast tissue and hence rather than being a poor prognostic marker, may reflect a protective response in the local stroma to limit the damage. One study has demonstrated that with time, breast cancer cells may become resistant to the inhibitory effects of IGFBP-3 (19). Our explant system is short term only and so unable to investigate such long term effects. It is possible therefore, that with time the breast tumour becomes insensitive to the effects of IGFBP-3. This may account for the inconsistencies found between epidemiological and *in vitro* data.

The possible protective *in vitro* role of IGFBP-3 in normal breast tissue may explain the high levels of IGFBP-3 mRNA detected in the breast tissue of healthy woman compared to those aged matched women with breast cancer. It would be interesting to follow up long term to see if any subsequently developed breast cancer and if so, whether the IGFBP-3 expression was affected.

5.9. Clinical potential

Targeting growth regulatory pathways in breast cancer has to date been an extremely effective way of treating breast cancer. Antagonism of the oestrogen and HER 2/neu receptors (304;305) have clearly shown beneficial effects and confirmed the biological role of the ER and HER 2 receptor in the development of breast cancer. Similarly, the demonstration that aromatase inhibitors are as effective as Tamoxifen in treating post-menopausal breast cancers suggests that there are many ways to manipulate a growth factor pathway.

IGF-I has been shown to be mitogenic and anti-apoptotic (306). Elevated levels of plasma IGF-I are known to be associated with an increased risk of breast cancer in pre-menopausal women (96). Oestrogen is well established in normal mammary development, however, is also associated with the development of breast cancer. Similarly, IGF-I is required for normal mammary development (83;84) and over expression results in mammary hyperplasia and neoplastic transformation (307). It is possible, therefore, that IGF-I participates in the malignant transformation of breast tissue much in the same way that oestrogen does.

A possible new anti-IGF therapy could involve inhibition of IGF-IR activation. Several attempts have been made to block this receptor using antibodies with varying results of IGF-I inhibition (308;309;309). It is also

possible that the IGF-I mitogenic effects may be mediated by receptors other than the IGF-IR. A more effective method of preventing IGF-I mediated mitogenesis maybe to use IGFBP-3 to neutralise its effects and also to have its own pro-apoptotic, anti-tumour effects. Studies have used IGFBP-1 in order to inhibit breast cancer growth in a xenograft model (310) and shown that growth of MDA-231 but not MCF-7 xenograft tumours were inhibited. This conflicts with *in vitro* studies where IGF-I stimulates MCF-7 breast cancer cells but not MDA-231 or -435 (309). Problems relating to the long half life of the IGFBP-1 used (PEG-BP-1) and poor tissue penetration were thought to be the cause of these results.

The therapeutic use of IGFBP-3 may be an alternative method of pharmacologically manipulating the IGF-I axis. Our results show that IGFBP-3 is unlikely to have an effect when used on its own. It may, however, be possible to achieve a therapeutic effect if used in combination with known cytotoxic agents such as doxorubicin or paclitaxel, particularly in ER negative tumours. There are no clinical trials yet established in this area.

Measuring serum IGF-I and IGFBP-3 levels may also provide a useful adjunct in women at increased risk of developing breast cancer and may provide useful information on prognosis should any subsequently develop breast cancer. Similarly, serum IGFBP-3 levels may be used to measure the tumours response to treatment. Other factors, however, such as proteases and other IGFBP-rPs contribute to the regulation of IGFBP-3 in the breast and further studies need to be performed to further elucidate their role. IGFBP-3 certainly plays an important role in maintaining tissue homeostasis, indirectly modulating IGF-I and independently modulating cell growth, however, the full complexities of the IGF system in the breast have yet to be fully understood. Hopefully, clinical studies may find an effective treatment strategy for use in treatment of breast cancer patients.

6. Conclusion

We have quantified the expression of IGFBP-3 in breast tissue and developed a viable system for keeping normal and breast tissue alive for up to 48 hours. The establishment of this novel, physiologically relevant, culture system has allowed us to study the effects of BP-3 on normal and malignant breast tissue. It is the first time that such effects have been quantified using this methodology. A larger sample size is needed, however, to confirm consistant patterns between individuals and to analyse the pathways involved in mediating BP-3's effects in the breast.

7. Appendix A

Tissue storage consent form '02

CONSENT FORM

TO BE USED WHERE AN OPERATION OR INVESTIGATION WILL INVOLVE REMOVAL AND STORAGE OF TISSUE, ORGANS OR BODY PARTS FROM THE PATIENT

Four copies of this form should be obtained.
One should be given to the patient, one filed in the hospital notes, one retained by the researcher and one given to pathology with the specimen.

Patient's details:
Name.....

Hospital number.....

D.O.B.....

Patient's condition requiring treatment
.....
.....

Intended operation / investigation
.....
.....

Tissue, organs or body parts involved
.....
.....

Tissue samples will be taken during your procedure, and sent to the hospital laboratories. This is routine practice essential for diagnosis and for planning further treatment. However in some cases more tissue will be removed than is needed for these tests. The aim of this form is to ask whether we may keep the remainder of the breast tissue that has been removed, to use in our research.

In our department we carry out research on breast disease. In your particular case, examples of the type of research we might plan include testing DNA and RNA (gene testing), growing cells from the sample in an incubator, analyzing the chemicals made by the cells in the sample, and finding out why the cells behave abnormally. Such research improves our understanding of these diseases.

By signing this form you will only be giving us permission to store your tissue for future research of this type. Any use that we want to make of it in the future will have first to be approved by our local Research Ethics Committee, which is an independent panel of experts who assess all research projects for safety, ethical acceptability and who protect patients' interests. Most of the work that we envisage will have no direct implications for your personal health. This may be because the tissue cannot be identified as yours, or it may simple be because of the type of research involved.

However in some circumstances we may wish to use your tissue which we can link back to you and your clinical record. Such research may have direct consequences for you or for your family. If it does, we would first ask the Research Ethics Committee and having obtained their approval for the research; we would always come back to you to explain the implications, and would then ask your consent to proceed. You would of course have the opportunity to refuse permission at that stage. If we do not believe our research will have implications for you or your relatives, and the Research Ethics Committee approves the research without your further consent, then we will not ask your permission.

Further details of the type of research we do are on the other side of this page, and will be explained to you by the doctor signing the form.

The information obtained from our research could be published in scientific journals and discussed at scientific and medical conferences. It would b completely anonymous. If we decided that it would be helpful to publish or discuss information about your case alone, it would still be anonymous but we would not go ahead without obtaining your consent again.

There is always a possibility that tissue, which cannot be linked back to you, may be used in the commercial development of medical technology. We would only ask for your consent to such development in relation to your tissue if for any reason it could be linked to you.

You do not have to agree to the storage of your tissue. You are free to decide not to participate.

I confirm that I have explained the nature of the tissue, organs or body parts that it is intended to remove. I have explained that they may be stored for use at a later date, in research as well as for diagnosis. I have explained this in terms which, in my judgment are suited to the understanding of the patient and/or guardian if the patient is a child.

Signature.....
Date.....

Name of practitioner seeking consent
(PRINT).....

FURTHER DETAILS OF THE PLANNED RESEARCH

Breast tumours are growths arising from the glands in the breast, and in the first instance the best treatment is to remove them surgically. You will shortly be undergoing an operation to remove your tumour, and this will be taken to the Pathology Department who will cut the tumour into very thin sections so that it can be examined under the microscope. This is an important part of your investigation as it will give your doctors detailed information about which will help them to provide you with the best treatment. However, a lot of the tissue is then left over, and we store this in the Pathology Department. We are very interested as to how these tumours start in the first place. We believe it is probably due to a change in the DNA, the information molecule at the center of every cell. Our research studies examine the cells of breast tumours to see how they are different from normal cells, and what makes them act as tumours. To this end, we may look at different types of chemical within the tumour, including DNA, RNA and protein molecules, which we can compare with normal tissue. We may also look at the way that these tumours make hormones when they are in test tubes, and see what controls their hormone production and also what makes the cells divide. No information relating to you personally will be used, but we may look at details such as your sex, age, the size of the tumour, and the particular abnormal cells. If we find any changes within the tumour, we my need to re-contact you for a blood sample or we may take one now if blood samples are taken as part of your current treatment. This would merely be to compare the results in the tumour to your blood to see whether the changes within the tumour were significant, and this would have no relation to your particular disease.

TO BE COMPLETED BY THE PATIENT/PARENT/GUARDIAN

Please read this form carefully. You will be given a copy.

If there is anything that you do not understand about the explanation and information you have been given, or if you want more information, you should ask the doctor before you sign the form.

If you understand the explanation and agree to the proposed plan, please print your name and sign in the space indicated below. Please note that you will also be asked to sign a separate consent form for the operation or investigation itself.

1. I understand that I am consenting for my tissue to be stored for research of a particular kind.

2. I understand that any further use of my tissue in the future for research of this kind will be approved by the local Research Ethics Committee.

3. I understand that if our research is thought likely to have any implications for me or my family, you will explain them to me, and ask my specific consent before proceeding with it.

4. I understand that if it is believed that our research will have no such implications, and if the Research Ethics Committee has approved the research, I will not be asked for further permission.

5. I understand that I will be asked to give consent for the commercial use of any tissue that can be linked back to me.

Signature.....Date.....
.....

Name .....Relationship: Patient/Parent/Guardian/Other*
**(please delete as necessary)*

This form should be witnessed by another person independent from the doctors looking after the patient. This could for example be a relative or member of the nursing staff.

Witness
signature.....Date.....

Name (PRINT).....Relationship.....

Where relevant, a translator should sign below to confirm they have interpreted the information on this completed form for the above patient in terms which they believe he or she can understand.

Translator
signature.....Date.....

Name .....Language used.....

Name and address of the Principal Investigator responsible for storing the patient's material (please PRINT):

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