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Correlation of Plasma Osteopontin Levels and 3D US Imaging with Tumor Response to Neoadjuvant Therapy

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Correlation of Plasma Osteopontin Levels and 3D US Imaging with Tumor Response to Neoadjuvant Therapy

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Abstract

Locally advanced breast cancer (LABC) is an aggressive form of pre-metastatic breast cancer, with an expected five-year survival rate of 30-42%. The current standard of treatment is neoadjuvant therapy; however there is a limited ability to monitor tumor response to this treatment. This study looks at two innovative approaches to monitoring tumor response: measurement of plasma osteopontin (OPN) levels and three dimensional ultrasound imaging (3D US). Using our laboratory's in house ELISA, we found that 39% of LABC patients had plasma OPN levels elevated above the normal healthy range of 123ng/ml (Bramwell,2006). Over treatment this percent increased, such that at final cycle, 63% patients had elevated OPN levels. Plasma OPN levels at the final two cycles of treatment were significantly different from early levels ($p<0.0001$). Furthermore, there was a trend towards an association between baseline and final OPN levels, and final response to treatment. Our study showed that there was no correlation between 3D US tumor volume with final tumor volume (as determined by pathology) after surgery ($r=-0.215$; $p=0.407$). However, we did see a strong statistical correlation between final tumor volume (from pathology) and final clinical estimation of tumor volume ($r=0.943$;$p=0.005$). Finally no correlation was observed between OPN levels and 3D US tumor volume ($r=0.004$; $p=0.987$). These novel findings show that plasma OPN level may have promise with respect to monitoring tumor response to neoadjuvant chemotherapy. Future studies with a larger patient population would be indicated.

Keywords: Locally Advanced Breast Cancer, Neoadjuvant Therapy, Osteopontin, Three Dimensional Ultrasound

Dedication

I would like to dedicate my work to my late Nana, Emma Caria and the wonderful patients who were part of this study. My Nana passed away seven years ago from breast cancer, but her love and belief in me has been a constant presence and motivation for me over the past two years of my research. To all the patients and their families, this study could not have happened without your support and your willingness to help advance research and we are truly grateful.

Acknowledgments and Co-authors

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List of Abbreviations

3D US	3 Dimensional Ultrasound
2D US	2 Dimensional Ultrasound
BSA	Bovine Serum Albumin
CR	Complete Response
ECM	ExtraCellular Matrix
ELISA	Enzyme-Linked ImmunoSorbant Assay
GST	Glutathione S-Transferase
LABC	Locally Advanced Breast Cancer
M	Metastasis
mRNA	messenger Ribonucleic Acid
N	Lymph Node
NR	No Response
OPN	Osteopontin
PD	Progressive Disease
PR	Partial Response
PI3K/Akt	Phosphatidylinositol 3-kinase/protein kinase B
r	Radius
RECIST	Response Evaluation Criteria In Solid Tumors
RGD	Arginine-Glycine-Aspartate
SVVYGLR	Serine-Valine-Valine-Tyrosine-Glycine-Leucine-Arginine
T	Tumor
uPA	urokinase-type Plasminogen Activator
π (Pi)	3.14
μ	Micro

Chapter 1: Introduction

1.1 Breast cancer

Breast cancer is the most commonly diagnosed cancer in Canadian females and will affect 1 in 9 women in their lifetimes. Although there has been a consistent decrease in incidence and death rates since 1969, this disease is still highly prevalent in Canada today. In 2007, 22,300 women were diagnosed with breast cancer and another 5,300 women succumbed to the disease (Canadian Cancer Society, 2007). Although breast cancer is predominantly a female disease, of the new cases presented, 170 were male breast cancers (Canadian Cancer Society, 2007). In most breast cancer cases, death is not the result of the primary breast lesion, but occurs when the tumor metastasizes. Once metastasis has occurred, treatment options become limited and cure unattainable, which is why research focusing on tumor progression is of importance.

1.2 Tumor progression and metastasis

Metastasis is the spread of cancer cells from the site of primary tumor growth to distant organs. In order for a metastatic tumor to become clinically detectable, a series of sequential steps must occur. Initially, a primary tumor mass must develop and from this mass, tumor cells then detach. Specific changes occur within both the tumor cells and the tumor microenvironment, that promote detachment and migration of the cells (Fantozzi, *et al.*, 2006). These changes enable the transformed tumor cells to degrade the surrounding basement membrane and extracellular matrix (ECM), enhancing the opportunity for the cells to move to different tissues. Once the ECM is degraded, the tumor cells can intravasate into nearby lymphatic or blood vessels. Once in the circulatory system, tumor cells must survive, avoid immune attack and disseminate to the

distant organ (MacDonald, *et al.*, 2002). Surviving tumor cells arriving at a distant organ extravasate into the new tissue, by again degrading the basement membrane and ECM (Sahai, *et al.*, 2007). Once in the new tissue, the tumor cell may be able to colonize and grow with the support of angiogenesis, defined as the formation of new blood vessels, and eventually forms the metastatic cell deposit.

When breast cancer is diagnosed and treatment commenced before the cells have metastasized, prognosis is more favorable. However, when a diagnosis occurs after the tumor has spread, response to treatment and overall survival is far less favorable (Jemal, *et al.* ,2008; Chambers, *et al.*, 2000). Jemal *et al.* (2008) showed that when cancer is diagnosed with the presence of distant metastasis, the five year survival rate is decreased across several types of cancer, compared to a diagnosis of a localized cancer. Patients with distant disease receive treatment to prolong disease stability, but are no longer curable. The clinical staging of breast cancer attempts to take into account tumor progression and metastasis as a reflection of tumor aggressiveness, so that individuals can be treated accordingly.

1.3 TNM staging

The most common classification for breast cancer staging is known as the TNM staging classification system. This system categorizes the extent of a patient's breast cancer based on tumor size (T), lymph node involvement (N) and metastasis (M) (Benson *et al.*, 2003). These characteristics are listed in detail in Table 1.1. The "T" designation reflects parameters such as the size of the primary tumor (measured clinically and pathologically after it is resected), and involvement of anatomically adjacent structures (skin or chest wall).

Primary tumor (T)	
T_x	Primary tumor cannot be assessed
T_0	No evidence of primary tumor
T_1	Tumor is $\leq$ 2 cm in greatest dimension
T_2	Tumor is > 2 cm but not > 5 cm in greatest dimension
T_3	Tumor is greater then 5cm in greatest dimension
T_4	Tumor of any size with direct extension into a)chest wall b) skin
Regional Lymph Nodes (N)	
N_x	Regional lymph nodes cannot be assessed
N_0	No regional lymph node metastasis
N_1	Metastasis in movable axillary lymph node
N_2	Metastasis in fixed axillary lymph node
N_3	Clinically detectable metastasis in ipsilateral axillary lymph node
Distant Metastasis	
M_x	Distant metastasis cannot be assessed
M_0	No distant Metastasis
M_1	Evidence of distant metastasis

Table 1.1 TMN clinical staging guidelines. Adapted from Singletary. *et al.*, 2002

The "N" designation reflects parameters such as the number of lymph nodes involved by tumor, the size of the intranodal tumor deposits, the location of the involved nodes and fixation of the involved nodes (Benson *et al.*, 2003). As the number of involved lymph nodes or the size of nodal deposits increase, staging score also increases. Detection of tumor deposits in the lymph nodes can be an indicator of propensity for disease spread, even when distant metastasis is not yet clinically detectable, thus making prognosis worse. Finally, the "M" designation reflects the presence or absence of distant metastasis in organs such as the brain, liver or bone. The presence of disease in a distant organ represents a much worse prognosis for the individual and can upstage a tumor (to stage IV) no matter what the state of the other two, T and N, components.

Stage is ultimately described as stage I, II, III, and IV, based on combinations of the TNM characteristics. Table 1.2 illustrates how the various T, N and M designations come together to give a stage I-IV. A higher stage number describes a more invasive cancer and therefore a more extensive disease. Moving from stage I to III the tumor size increases and regional lymph nodes and/or organs adjacent to the primary tumor may have tumor involvement. A stage IV cancer is one that has spread distantly to other organs.

Stage Group	T	N	M
I	T1	N0	M0
IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

Table 1.2 TNM stage grouping for breast cancer.

1.4 Locally advanced breast cancer

Locally advanced breast cancer (LABC) is the designation given to the most advanced stage of nonmetastatic breast cancer. In this form, the tumor has progressed locally, but has not yet spread beyond the breast and the regional lymph nodes (Giordano, 2003). TNM staging guidelines define LABC as any stage III disease, and as such these patients often present with a variety of clinical scenarios (Green *et al.*, 2002). In most LABC cases the common shared characteristic is the presence of a large primary tumor, which may be invading adjacent structures. There can be advanced regional nodal disease, involvement of the chest wall or skin, and/or the clinical presentation of inflammatory carcinoma (Valero *et al.*, 1996).

This form of breast cancer represents some of the most aggressive breast cancers seen today (Chia *et al.*, 2008). The majority of LABC patients will eventually develop distant metastasis despite aggressive treatment, providing a clinical challenge to treating these individuals (Giordano, 2003). Another major clinical problem is the high rate of disease recurrence and the poor survival rate, as these patients have a higher chance of having distant sub-clinical metastases at the time of initial diagnosis (Giordano, 2003; Chia *et al.*, 2008).

With the increasing use of mammographic screening, the incidence of LABC cases has decreased, however this condition still accounts for approximately 15% of all breast cancer cases (Jemal *et al.*, 2004). Prognosis for LABC depends on initial tumor size, lymph node involvement and the presence or absence of inflammatory breast cancer. The five year survival for LABC patients is only 30-42%, such that treatment must not only

be aggressive, but must allow for close and accurate monitoring of the tumor response (Jemal *et al.*, 2004).

Treatment for this form of cancer is multimodal, involving a combination of chemotherapy, surgery and radiation. Due to the large size of the tumor at diagnosis, immediate surgery is usually not feasible for most LABC patients, such that they instead routinely receive neoadjuvant chemotherapy first, followed by surgery and radiation.

1.5 Neoadjuvant treatment for LABC

In the past, LABC was initially treated with radical surgery and/or radiation. However, many patients still developed distant metastases within 24 months of this treatment and several experienced locoregional failure (Valero *et al.*, 1996). Valero *et al.* (1996) showed that after radical surgery alone, up to 60% of the LABC patients had developed a local recurrence, suggesting that local control was inadequate. Local control is required because the initial size of the primary tumor, the number of involved regional lymph nodes and the invasion of the tumor into breast muscle and skin, are all factors that lead to increased recurrence rates in this disease population. Optimal management of LABC was therefore found to require aggressive treatment in order to prevent further disease progression. Today, the widely accepted treatment for LABC is neoadjuvant therapy. Neoadjuvant therapy differs from standard adjuvant therapy (which is commonly used for early breast cancer treatment) in that patients have systemic chemotherapy prior to surgery and radiation (Giordano *et al.*, 2003). Neoadjuvant therapy has been shown to be as safe and effective as adjuvant therapy, which delivers chemotherapy after surgery has successfully excised the tumor (Kaufmann *et al.*, 2006). Survival rates for patients treated with either regimen are shown to be similar, therefore making either treatment

approach reasonable for a patient with operable breast cancer (Gralow *et al.*, 2008). However, in patients with initially inoperable, locally advanced tumors, neoadjuvant therapy is used as early as possible in order to shrink the tumor to a size more manageable surgically and to target any cells that may have begun to spread (Kaufmann *et al.*, 2006). The use of chemotherapy up front increases tumor response and thus facilitates the local control, which is gained through surgery and radiation (Gralow *et al.*, 2008). The primary goal of this aggressive therapy for locally advanced breast disease is to optimize cure and improve overall survival. With the addition of up front chemotherapy, clinicians have noted that 3%-10% of LABC patients show no pathological presence of invasive tumor remaining in the breast or regional lymph nodes at the time of surgery (Cocconi *et al.*, 1990; Swain *et al.*, 1987). This subgroup of patients have a vastly improved prognosis over the remaining LABC cohort.

Although there are several chemotherapy regimens which are standard in neoadjuvant therapy, most guidelines, including guidelines from the National Comprehensive Cancer Network (NCCN) for breast cancer treatment, indicate that an initial anthracycline-containing regimen is preferred (Kaufmann *et al.*, 2006; Schwartz *et al.*, 2002; Shenkier *et al.*, 2004). Furthermore, studies have concluded that the addition of a taxane to the anthracycline regimen increases the chances of a complete pathological response and leads to improvement in overall survival in node-positive breast cancers (Esserman *et al.*, 2004; Taghian *et al.*, 2008). Based on guidelines from the BC Cancer Agency and Cancer Care Ontario, this regimen, using treatment with an anthracycline followed by a taxane, is currently standard therapy in the province of Ontario and throughout Canada for the management of LABC.

Once patients have received initial systemic therapy, the next step is to treat locally by means of surgery and radiation. Gaining local control of the tumor is important in the treatment for breast cancer, because distant metastatic disease can result from a local breast recurrence (Vicini *et al.*, 2008). Several Phase II studies have shown that when primary chemotherapy is followed by both surgery and radiation, there is a higher rate (up to 85%) of local control, compared to using either surgery or radiation alone after chemotherapy (Valero *et al.*, 1996). In LABC patients treated either with surgery or radiation alone, the risk of local recurrence is in the order of 30-50% (Taghian *et al.*, 2008).

Historically, radical mastectomy was used as a means of treating LABC patients, however the local failure rate after surgery alone was 60% (Valero *et al.*, 1996). The current surgical standard for the treatment of LABC as determined by the BC Cancer Agency and Cancer Care Ontario, is that after neoadjuvant chemotherapy shrinks the primary tumor to an operable size, patients undergo a modified radical mastectomy. Breast conserving surgery is currently not considered to be standard treatment, however some studies suggest that after shrinking the tumor with initial chemotherapy, breast conserving surgery can be an option for a carefully select group of patients (Chia *et al.*, 2008; Singletary *et al.*, 1992). Large scale studies are required to more closely look at breast conserving surgery before it is to be considered as a standard approach. However, it is undisputed that surgical excision of the primary tumor is associated with significantly longer survival in breast cancer patients, indicating the importance of this aspect of treatment for these patients, even after having a response to chemotherapy (Fields *et al.*, 2007).

In addition to surgery, to optimize local control, radiation therapy is offered as further treatment for LABC patients. Radiation therapy is performed to ensure that any residual microscopic disease which may be left behind after surgery is eradicated. Studies have shown that the use of radiation therapy after surgery increases long term survival and decreases locoregional recurrence rates (Pierce *et al.*, 2008).

1.6 Benefits of neoadjuvant therapy

One of the major advantages of neoadjuvant therapy for patients with LABC is that up front chemotherapy can downstage large, inoperable tumors, making them operable. Another advantage that comes with this form of treatment is that with early systemic chemotherapy, vasculature near the primary tumor is intact and has not yet been affected by surgery and radiation, thus allowing more efficient delivery of the drugs to the primary tumor. Also, exposing sub-clinical micrometastases to early therapy can help improve outcome and survival (Chia *et al.*, 2008; Giordano, 2003; Gralow *et al.*, 2008).

Furthermore, when treating with neoadjuvant therapy, the primary tumor remains in the breast, providing the opportunity to study the efficacy of the chemotherapy on the tumor, as well as, allowing assessment of the response to therapy throughout treatment (Giordano, 2003).

1.7 Current methods of monitoring tumor response to neoadjuvant therapy

When treating with neoadjuvant therapy, the primary tumor can be closely monitored to determine if the treatment is working. The primary tumor's response to chemotherapy is an important predictor of the overall survival rate for LABC patients and therefore must be accurate. However, there are no current standard protocols for assessing the clinical response to neoadjuvant therapy, other than physical examination estimations of

the breast mass over the course of chemotherapy (Taghian *et al.*, 2008). As defined by the World Health Organization/International Union Against Cancer as well as the Response Evaluation Criteria in Solid Tumors, a clinical complete response to neoadjuvant therapy occurs when there is a complete disappearance of all clinically detectable disease in the breast and regional lymph nodes (Taghian *et al.*, 2008). However, there are problems associated with this method of physically measuring the tumor mass during treatment. Inter-individual variation among examiners can lead to different measures in response (Taghian *et al.*, 2008). Another major problem is that the correlation between clinical response and the final pathological response is not always accurate, especially when there is an extensive change in the primary tumor (eg. extensive necrosis, scarring/fibrosis), making measurement difficult.

Most patients, when treated with neoadjuvant chemotherapy, do have a response to treatment. Approximately 10%-20% of patients achieve a clinical complete response and 50%-60% will achieve a partial response to neoadjuvant therapy. However, the literature would indicate that in cases where a clinical complete response is assessed, approximately one-third of these patients are later found to still have pathological evidence of residual disease (Giordano, 2003).

Postoperative pathological assessment of the tumor thus is another important component of treatment. This assessment provides the most accurate determination of how responsive the primary tumor was to chemotherapy, based on the histological presence or absence of residual invasive tumor. The extent of the tumor's response is important for determining the overall survival for the patient (Taghian *et al.*, 2008). Guidelines commonly accepted today for monitoring response of the primary tumor to

treatment, use unidimensional measurements and the sum of the largest diameters of the target lesion for response evaluation (Therasse *et al.*, 2000). These guidelines have been validated by the Response Evaluation Criteria in Solid Tumors Group (RECIST) (Therasse *et al.*, 2000). The RECIST guidelines for the response of a target lesion indicate that a complete pathological response occurs when there is a disappearance of the entire primary lesion. When the tumor has decreased by at least 30% of its original size, it is considered to be a partial response and if there is at least a 20% increase in the size of the lesion, it is considered to be progressive disease (Therasse *et al.*, 2000). The disease is considered to be stable, or the individual has had no response to treatment, when there is insufficient shrinkage or growth to be classified in one of the above response categories.

After treating the tumor with chemotherapy, the excised specimen may be difficult to interpret grossly and histologically. Prognostic factors may be altered as a result of treatment (Pinder *et al.*, 2007). Common after neoadjuvant therapy is the presence of a central fibrotic area of scarring, which indicates the site of the original tumor (Pinder *et al.*, 2007). Scarring and regions of dense fibrosis however, make assessing residual tumor size difficult, especially when there are several foci of residual carcinoma (Symmans *et al.*, 2007). Although there is difficulty in assessing the response of a primary tumor to neoadjuvant therapy, both clinically and pathologically, it is important in further management decisions and in predicting the patient's overall prognosis and survival. Patients that have a complete pathological response to this treatment have a better outcome and longer survival rates compared to non-responders (Giordano *et al.*, 2003; Taghian *et al.*, 2008). Since only one half, to up to two thirds of clinical complete

responses will be confirmed pathologically, it is important that new approaches to monitoring tumor response during treatment are investigated (Taghian *et al.*, 2008).

In this study, we use two novel approaches for monitoring tumor response to neoadjuvant therapy: the measurement of plasma osteopontin (OPN) levels and three-dimensional ultrasound (3D US) measurement of tumor volume, with both to be assessed at each visit during the course of therapy. The question addressed in this study is if either or both of these methods provide a more reliable method for assessing *in vivo* response of the tumor to treatment.

1.8 Osteopontin

Osteopontin (OPN) is a secreted, integrin-binding phosphoprotein. It is expressed in the form of a nascent protein and is modified by post-translational events, which result in cell-type and condition specific variations in OPN structure (Sodek *et al.*, 2000).

OPN is expressed by several normal tissues and cell types such as the kidney, bone, brain, dentin, cementum, vascular tissue, hypertrophic cartilage, bone-marrow-derived metrial gland cells, epithelia in mammary, salivary and sweat glands and activated macrophages and lymphocytes (Wai *et al.*, 2004;Tuck *et al.*, 2001). OPN is also found in various biological fluids including blood, milk, urine and seminal fluid (Wai *et al.*, 2004). OPN is elevated during the development of different tissues and in various reactive states such as inflammation, vascular remodeling, bone remodeling and immune responses (Tuck *et al.*, 2001; Rangaswami *et al.*, 2006). In addition, OPN expression is found to be associated with several disease conditions, including kidney disease, atherosclerosis and cancer (Rangaswami *et al.*, 2006). The synthesis of OPN can be regulated at both the post-transcriptional and post-translational level and OPN from different cellular sources

may have different structural characteristics reflective of their role (Rangaswami *et al.*, 2006). OPN expression is regulated by various hormones, cytokines and growth factors, which can affect its rate of gene transcription, mRNA processing, stability, translation and post-translational modifications (Sodek *et al.*, 2000).

OPN is rich in aspartate, glutamate and serine amino acid residues and its protein backbone contains several conserved structural elements and functional domains, including the heparin and calcium binding domains, extra-cellular matrix adhesion domains, domains for glycosylation and phosphorylation, a thrombin cleavage site, and an RGD (Arg-Gly-Asp) integrin-binding domain. Figure 1.1 shows the structure of OPN and its conserved structural elements. The RGD domain binds $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ cell-surface integrins (Wai *et al.*, 2004). Downstream from the RGD domain is the thrombin cleavage site. This protease-hypersensitive site is where thrombin cleaves OPN into two fragments, revealing the integrin binding domain on the N-terminal fragment, and on the C-terminal fragment, the CD44-binding site. This cleavage also reveals the Serine-Valine-Valine-Tyrosine-Glycine-Leucine-Arginine (SVVYGLR) amino acid sequence, which binds $\alpha_9\beta_1$. Functionally, OPN has been shown to mediate cell adhesion, chemotaxis, macrophage-directed interleukin-10 suppression, stress-dependent angiogenesis, prevention of apoptosis and anchorage–independent growth of tumor cells (Wai *et al.*, 2004).

1.8.1 Role of OPN in tumor progression and metastasis

Tumor progression is a multistep process that involves genetic and epigenetic changes. These changes provide cells with a growth advantage, which allows them to progress to a malignant phenotype.

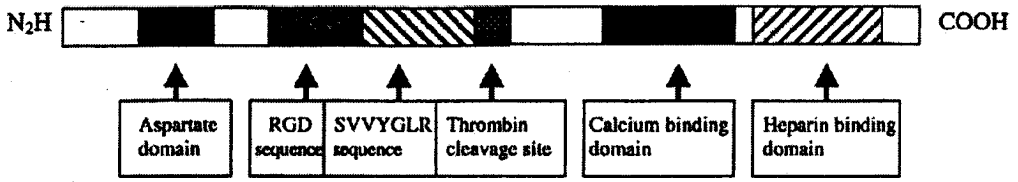

Figure 1.1 Molecular structure of OPN. Integrin binding sequences and proteolytic cleavage sites are shown. GRGD integrin binding sequence, SVVYGLR integrin binding sequence, thrombin cleavage site, the calcium binding domain and the CD44-heparin binding domain. The OPN terminal N_2H and COOH ends are conserved. Figure adapted from Wai, *et al.*, 2004.

Tumor progression is believed to occur as the cell begins acquiring six essential characteristics, known as the "hallmarks of cancer" (Hanahan *et al.*, 2000). These characteristics include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenisis and tissue invasion and metastasis.

Evidence has accumulated showing that OPN is associated with the aggressiveness of several cancer types, including breast, prostate, hepatocellular, and colon cancers and that this protein is suggested to play an important role in various aspects of malignancy, particularly those involved in tissue invasion and metastasis (Cook *et al.*, 2005).

OPN was first characterized in 1979 by Senger et al, as a transformation-associated protein, which historically provided the first link between OPN and cancer (Senger *et al.*, 1979; Rittling *et al.*, 2004). Since then, studies have shown that OPN expression can render cells more tumorigenic and/or metastatic. Furthermore, when OPN expression is down-regulated by an antisense approach, there is reduced cell growth in soft agar and also reduced growth of the primary tumor *in vivo* (Rittling *et al.*, 2004). Increased OPN mRNA levels, as well as protein levels, have been detected in a number of carcinomas such as colon, pancreas, breast and lung, when compared to the corresponding normal tissue (Brown *et al.*, 1994; Coppola *et al.*, 2004).

In our laboratory, we have shown that OPN affects breast cancer cells, both *in vitro* and *in vivo*. *In vitro,* we have shown that OPN can support cell adhesion and can induce cell migration and invasion of mammary epithelial cells, indicating OPN's functional role in the progression of breast cancer (Tuck *et al.*, 2001). *In vivo* studies have also shown that OPN increases tumor aggressiveness. When MDA-MB-468 human breast cancer

cells transfected to express OPN, are injected into the mammary fat pad of nude mice, there is increased lymph node metastases, increased lymphovascular invasion and increased lung micrometastases compared to MDA-MB-468 cells that express mutant OPN (Allan *et al.*, 2006). Downstream signals induced by OPN have also been identified, which are important in regulating tumor aggressiveness and invasive behavior. One of the pathways that OPN activates is the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, which has been shown to prevent apoptosis and provide a survival advantage for murine B cells (Wai *et al.*, 2004). Other pathways that OPN can influence (and therefore affect tumor cell behavior), include growth factor receptor pathways (eg. hepatocyte growth factor and its receptor c-Met), and cell proteases/receptor pathways, such as urokinase-type plasminogen activator (uPA) and its receptor (uPAR) (Furger *et al.*, 2001). Furthermore, OPN can increase tumor angiogenesis and increase tumor cell survival by inhibiting apoptosis (Cook *et al.*, 2005).

OPN interacts with various cell surface receptors, such as integrins and CD44, which leads to the activation of various signal transduction cascades. This activation of secondary messengers can result in changes in gene expression, which will ultimately affect the behavior and properties of the tumor cell (Allan *et al.*, 2006; Newham *et al.*, 1996; Tuck,A.B. 2001 (36)). These changes have been shown to affect malignancy by increasing invasion, adhesion, migration, survival, angiogenesis and metastasis (Tuck *et al.*, 1999; Tuck *et al.*, 2000; 71 Tuck *et al.*, 2001; Cook *et al.*, 2005; Furger *et al.*, 2003; Xuan *et al.*, 1995).

Using 21NT mammary carcinoma cells transfected to over-express OPN, our laboratory showed that when compared to the 21NT control cells with low OPN

expression, several genes were found to be differentially expressed (Cook *et al.*, 2005). These OPN regulated genes can be functionally classified into the six categories of the "hallmarks of cancer" (Cook *et al.*, 2005). By showing that OPN can induce multiple changes in gene expression that affect the six hallmarks of cancer, it is clear that the effects of this one gene on various aspects of malignant cell behavior can be broad and far-reaching.

1.8.2 Clinical significance of osteopontin

OPN expression has been shown to be associated with cancer and metastasis. Early work showed that a variety of tumor cells have increased OPN levels compared to their corresponding normal tissues (Brown *et al.*, 1994; Coppola *et al.*, 2004). Specifically, breast tumors have been shown to have increased OPN immunopositivity compared to levels in benign breast lesions (Bellahcene *et al.*, 1995). Previous work has revealed that OPN can be secreted by breast cancer cells themselves (Tuck *et al.*, 1998). Work by our laboratory, as well as other groups, has shown that the elevated levels of OPN found in primary tumors is correlated with a poor patient prognosis (Tuck *et al.*, 1997; Tuck *et al.*, 1998; Rudland *et al.*, 2002). Also, in multiple tumor types, an increase in OPN expression is significantly correlated with tumor stage (Coppola *et al.*, 2004). Rudland *et al.* (2002) showed that in primary tumors, as the level of OPN increased, so did histological grade. Furthermore, the elevated levels of OPN in the primary tumors of breast cancer patients has been shown to be significantly associated with a more aggressive tumor phenotype, as well as with tumor progression (Tuck *et al.*, 1997; Tuck *et al.*, 1998). Finally, it has been shown that overall survival and disease free survival is associated with the level of breast tumor OPN (Tuck *et al.*, 1998; Rudland *et al.*, 2002).

In addition to detecting OPN in the primary tumor of cancer patients, OPN can also be detected in the blood of patients with various forms of cancer, such as breast, prostate, colon, lung, liver and stomach cancer (Bramwell *et al.*, 2006; Singhal *et al.*, 1997; Senger *et al.*, 1988; Hotte *et al.*, 2002). In order to measure the levels of blood OPN, our laboratory has developed a capture Enzyme-Linked ImmunoSorbant Assay (ELISA), which uses monoclonal mouse and polyclonal rabbit antibodies to measure the levels of OPN in blood plasma (Bautista *et al.*, 1996; Singhal *et al.*, 1997). In healthy women, the levels of plasma OPN have been shown to remain consistently low over time and are unaffected by cyclical changes in hormones over the menstrual cycle (Bautista *et al.*, 1996). However, plasma OPN levels are found to be elevated in metastatic breast cancer patients (Singhal *et al.*, 1997). In addition, higher baseline levels of plasma OPN in metastatic breast cancer patients are associated with a worse prognosis and increased tumor burden (Bramwell *et al.*, 2006; Singhal *et al.*, 1997). Singhal *et al.* (1997) have shown that elevated plasma OPN levels are not only associated with decreased survival, but with the number of sites of tumor involvement. Also, in metastatic breast cancer patients monitored by serial OPN blood levels, survival decreases (despite treatment) as plasma OPN levels increase over time (Bramwell *et al.*, 2006).

Plasma OPN may thus have both a prognostic and a predictive role, making monitoring plasma OPN levels from breast cancer patients throughout treatment and over disease course potentially useful. The association between elevated plasma OPN and decreased survival suggests that OPN could possibly be used to predict aggressive tumor behavior. LABC patients are known to have a poor prognosis and despite treatment, still have disease relapse and develop metastasis. The significance of measuring OPN in these

individuals is that this is the first time that plasma OPN levels will be measured at baseline and then sequentially through treatment, for patients bearing a primary tumor. Measuring plasma OPN levels over treatment may potentially provide information with respect to patient response. Being able to more accurately monitor response to neoadjuvant therapy may lead to better management of these patients.

1.9 Three dimensional ultrasound imaging

In the past few decades, advancements in imaging techniques have been made, allowing for more precise and accurate diagnosis and staging of disease, including cancer. Ultrasound imaging has long been used, but only in this past decade has it become a reliable imaging tool, due to developments in image quality and advancements in technology. One of the greatest advancements in ultrasonography has been the development of the three dimensional ultrasound (3D US) imaging system. This system allows for high resolution, three dimensional images to be acquired in a rapid, inexpensive and non-invasive manner (Fenster *et al.*, 2000; Fenster *et al.*, 2004).

3D US imaging provides a significant advantage over conventional 2D US imaging. With conventional imaging, a series of 2D images are obtained and in order to generate an estimate of the three dimensional structure, the ultrasound technician has to mentally combine these images (Fenster *et al.*, 2001). Therefore, the resulting 3D estimates of tumor size are largely subjective and vary between users. This method is not only time-consuming but because of its subjectivity, it often leads to inaccurate measurements. In order to calculate a tumor volume from the 2D images, an idealized shape has to be assumed and simple measurements of height, length and width are calculated, resulting in

low accuracy and high variability (Fenster *et al.*, 2001). In order to compensate for the limitations of conventional 2D US, a three dimensional system has been developed.

Previous work has shown that 3D US systems allow for a non-invasive method of localizing a breast tumor (Cash *et al.*, 2007). In addition, preliminary experiments conducted in Dr. Fenster's laboratory (Robarts Research Institute, London, Ontario, Canada) using this unique 3D US system to ultrasound simulated agar "tumors" has shown the ability to measure known volumes within 3% (DeJean *et al.*, unpublished data). Furthermore, this ultrasound system has been shown to be able to properly visualize and measure patient's breast tumors clinically (DeJean *et al.*, unpublished data).

Accurate 3D US imaging makes use of a traditional transducer attached to an automated scanning system, which acquires a series of 2D images. These images are then reconstructed by the computer system to generate an objective 3D image of the complete structure. 3D US imaging is a user-friendly imaging modality that allows for 3D images to be acquired rapidly. In order to image LABC patients' tumors over the course of therapy, we required a system that was portable so that it could easily be brought into the clinic. Our 3D US system is a portable, cost effective and user-friendly system, which is the size of a laptop. This system was developed by Dr. Aaron Fenster, by integrating a terason t3000 portable US system with 3D scanning hardware and software (also uniquely developed by Dr. Fenster) (Fenster *et al.*, 2001).

This system supports the use of high frequency transducers, thus allowing for high resolution imaging. The transducer that we use is the Terason 7L3 7-3MHz Linear Wideband US probe. Figure 1.2 shows this 3D US system. This system operates with a mechanical 'tilt'scanner to acquire 3D US tumor images by using a motor to tilt the

transducer and acquire a series of 2D images rapidly (Fenster *et al.*, 2001). The images are taken at regularly-spaced intervals of 0.5°, starting at the centre of the tumor, so that the whole volume of interest can be assessed (Fenster *et al.*, 2001; Ladak *et al.*, 2000). Figure 1.3 shows the method of scanning with arrows indicating motion of tilt.

Once the tumor has been scanned, immediate 3D reconstruction of the image can be completed in order to obtain the 3D representation of the tumor. From this representation, tumor volume can be determined, which is one advantage of the 3D system. To do this, 3D segmentation software has also been developed by Dr. Fentster (Fenster *et al.*, 2001; Ladak *et al.*, 2000; Wang *et al.*, 2003). This software enables manual segmentation of the tumor to generate a complete tumor volume. With manual segmentation, the 3D image is sliced into a series of uniformly-spaced, parallel 2D images, from which measurements are taken and used to accurately estimate the tumor volume (Fenster *et al.*, 2000). Both the number of slices taken and the slice interval (0.5-2.0mm) are determined by the total tumor size.

Integrating this portable system into the clinic allows for rapid sequential imaging of a LABC patient's tumor over the course of neoadjuvant therapy, which may provide a more accurate tumor volume from which response to treatment can be determined.

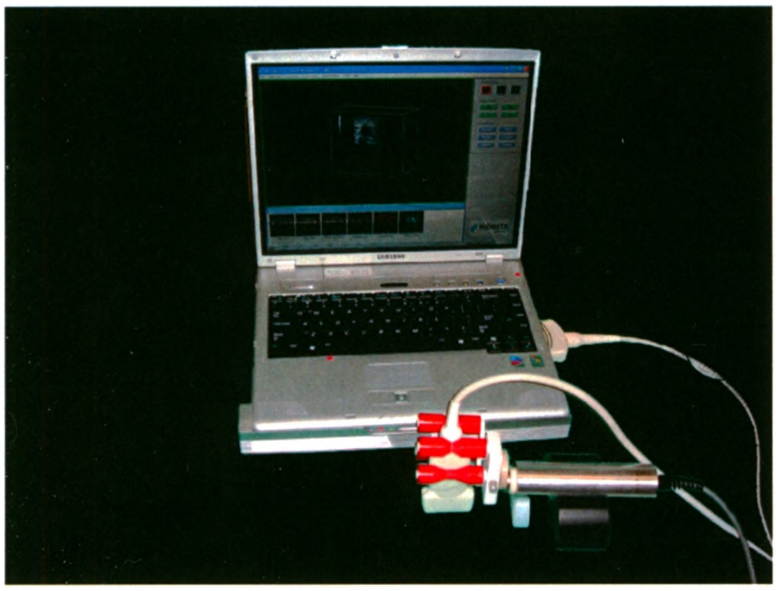

Figure 1.2 Terason t3000 3D US system. Portable ultrasound unit attached to a hand held scanner with attached transducer.

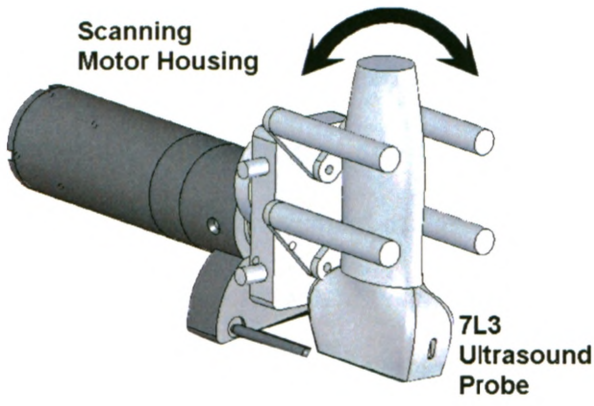

Figure 1.3 Schematic of the hand held "tilt" scanning system. Arrows indicate direction of the tilt scanning motion, starting at centre of tumor then moving completely to one side and then back to additional side. The hand held adapter attaches and holds the 7L3 transducer. (DeJean *et al.*, unpublished data).

1.10 Thesis Hypothesis and Objectives

The overall objective of my research work is to investigate the use of two new methods to monitor tumor response to neoadjuvant therapy for women with LABC. It is hypothesized that measuring plasma OPN levels and using 3D US imaging will provide reliable and convenient methods to more accurately do this. To achieve this, nineteen LABC patients at the London Regional Cancer Program were enrolled in this pilot study. Three major objectives of this work are as follows:

Objective One: Measure plasma OPN levels over the course of neoadjuvant therapy for all patients enrolled in the study.

Objective Two: Measure tumor volume with 3D US over the course of neoadjuvant therapy for all patients enrolled in the study.

Objective Three: Compare these measurements with clinical breast measurements and final tumor pathology.

1.11 Significance

LABC patients have a poor overall prognosis, due to their high risk of tumor recurrence and development of future metastasis. This study has the potential to help improve the clinical management of these patients. This translational study provides a first to quantify tumor growth *in vivo* through the use of 3D US imaging, while measuring the level of plasma OPN, to more accurately monitor tumor response to neoadjuvant chemotherapy. The expectation is that 3D US imaging and/or plasma OPN levels may be used to predict patients who will or will not respond to neoadjuvant chemotherapy. The significance of this study is that it may provide clinicians with real-time information about response to treatment and therefore help with the management

and treatment decisions for these patients. Through more accurate assessment of tumor response to neoadjuvant treatment, improved survival and prognosis may be possible.

Chapter 2: Materials and methods

2.1 Patients

Nineteen patients diagnosed with LABC, being treated at the London Regional Cancer Program, London, Ontario, Canada were enrolled into this study. Eighteen were female patients, with one LABC male patient. All patients had a histologically confirmed breast cancer and had to be eligible for neoadjuvant therapy. The decision to proceed with neoadjuvant therapy was made by the medical oncologist, following a diagnosis of LABC at the clinical exam consultation. Patients had to be between the ages of 18 and 80 years of age to participate in the study, excluding minors due to consent issues and those above 80 years of age due to increased toxicity with chemotherapy. In addition, patients also had to have no evidence of inflammatory breast cancer or metastasis at initial diagnosis. Baseline blood samples were taken for plasma OPN levels and baseline 3D US images were required from each patient, prior to the initiation of chemotherapy. Further, to be eligible for the study, a 3D US image had to be visible using the 3D US scanning system described below. This study was approved by UWO's Health Sciences Research Ethics Board (HSREB) and all patients had to provide written informed consent prior to enrollment.

2.2 Trial Design

Patients were seen by the medical oncologists at the London Regional Cancer Program prior to beginning neoadjuvant therapy and for the duration of treatment. The type of neoadjuvant systemic chemotherapy offered was determined by the patient's oncologist and comprised of an anthracycline followed by a taxane regimen. Patients were seen at each chemotherapy treatment visit, which occurred once every three weeks,

for eight cycles. 16 patients completed eight cycles of neoadjuvant chemotherapy. One patient died after two cycles of chemotherapy and two additional patients who, because metastases were discovered after enrollment in the study, only had a six course regimen. Patients were followed according to the guidelines of care for all LABC patients at the London Regional Cancer Program. This included a physical exam performed by the medical oncologist. This exam was done to obtain a clinical estimate of the tumor size, which was used to assess the response of the tumor to chemotherapy. This examination is the only standard for monitoring response and was repeated at all patient visits, as long as the tumor remained palpable. From this clinical examination, the largest dimension of the tumor size was used to estimate the tumor spherical volume. Volume was calculated using the formula $4/3\pi r^3$. Volume was used for the clinical measure of tumor response, in order to directly compare with the volume measure from 3D US imaging. In addition, patients also received routine blood work (blood count, biochemical screen), which was completed at baseline and at each chemotherapy treatment cycle (once every three weeks). In addition to standard investigations, patients enrolled in the study also had plasma samples collected for OPN measurement, and a 3D US image of the breast tumor, at baseline and again at each chemotherapy cycle. For patients that received all eight cycles of chemotherapy, a total of eight OPN samples and eight 3D US images (per patient) were obtained. After all courses of chemotherapy were administered, the patients underwent modified radical mastectomy to surgically remove the breast and residual tumor mass, as well as the axillary lymph nodes, which were then examined for the final pathology.

2.3 Final pathology

All patients, with the exception of the one male patient who died and the two metastatic patients, completed the treatment course (described above). Surgical specimens were sent for final pathological assessment, which was performed by the pathologists in London, Ontario. From this final pathology report, residual tumor size was obtained, in terms of number of invasive foci and largest dimension (diameter) of each invasive focus. From the largest dimension of each residual invasive focus, spherical volume was calculated ($4/3\pi r^3$). The final tumor volume was estimated and recorded as the sum of the volumes of each invasive focus. This final tumor volume was then used for comparison with the 3D *in vivo* US volume of the tumor, the estimate of the *in vivo* tumor volume by clinical examination, and plasma OPN level. Additionally, the response of the primary tumor to chemotherapy, using the RECIST guidelines, was determined for each patient. Tumor response to neoadjuvant chemotherapy was substratified as follows (Therasse *et al.*, 2000):

i) Complete pathological response: No evidence of residual invasive tumor

ii) Partial response: at least a 30% decrease in the sum of the largest dimensions of the target lesions

iii) No evidence of response/stable disease

iv) Progression of invasive tumor: at least a 20% increase in the sum of the largest dimensions of the target lesions

2.4 Plasma collection and OPN measurement

Blood samples were collected from all patients at baseline (prior to chemotherapy) and at each chemotherapy cycle treatment, for eight cycles with the exception of the male patient who died and the two patients who received only six courses of treatment (samples for OPN measurement collected at the same time as routine blood sampling for clinical testing). For one of the patient's, blood OPN levels were not obtained at baseline but were subsequently collected during the chemotherapy cycles. Blood samples for OPN measurement were taken by the hematology lab at the London Regional Cancer Program and collected in tubes with EDTA anticoagulant. Blood was then processed, in order to generate plasma samples, by centrifuging the tubes at 2,000 rpm for 10 minutes at room temperature. The plasma was then transferred into two 1.5mL Eppendorf tubes and centrifuged at 11,000 rpm for 3 minutes at room temperature. This was done to remove any cell debris and white cells from the sample. Plasma was then transferred to clean tubes and stored at -80° C until analysis.

Plasma was analyzed for OPN using our laboratory's in-house ELISA assay, which has been previously validated (Bramwell *et al.*, 2006; Singhal *et al.*, 1997). Our "capture" ELISA system makes use of a high affinity mouse monoclonal antibody, mAB53, which is specific for human OPN, and a rabbit polyclonal antibody which was developed against a recombinant human OPN-GST fusion protein (GST-hOPN), that also recognizes native human OPN (Singhal, 1997). 96-well immunoassay plates (Nalge Nunc International, Rochester, New York) were coated with mAb53 (100μL/well, 2.5μg/mL in 0.1 M sodium bicarbonate, pH 9.0), then blocked with 1% BSA in ST buffer (0.01 M Tris, pH 8.0, 0.15 M NaCl) with 0.05% Tween-20 (Bio-Rad, Mississauga, Ontario,

Canada), followed by washing (2 times, 400 µL) with ST-Tween buffer. 10 µL of patient plasma were then loaded onto the wells containing 90 µL ST-Tween buffer + 1% BSA and incubated for 90 min at 4°C for the capture of OPN. The wells are washed seven times with 400 µL ST-Tween buffer followed by incubations with rabbit anti-OPN antiserum (1 hour, 37° C), biotinylated goat anti-rabbit immunoglobulin antibody (Dako, Carpinteria, Ca) (1 hour, 37° C) and with streptavidin-conjugated with alkaline phosphatase (Jackson Immunological Laboratories Inc, West Grove Pa) (1/2000) for one half hour, which tagged the biotinylated goat anti-rabbit immunoglobulin antibody. Following each incubation step, wells were washed with ST-Tween buffer as above. Finally, the wells were treated with 100 µl of p-nitrophenol phosphate (img/ml in 100 M Tris, pH 9.0, 100 mM NaCl, 0.5 M $MgCl_2$; Sigma, St. Louis, MO) for 4-6 minutes at room temperature to allow for colour development. To stop the reaction, 50 µl of 0.5 M Na_2EDTA (pH 8.0) was added and the plate was then read using a Bio-Rad plate reader to quantify the signal. The signal is a colour change which is read at 405 nm; the measured optical density is directly proportional to the concentration of human OPN in the samples. Recombinant GST-hOPN was used as a standard, and internal controls of plasma were included for use in normalizing OPN values obtained from independent experiments. Plasma OPN measurements were compared at baseline with those throughout treatment for all patients.

2.5 3D US imaging and segmentation

3D US imaging of the breast tumor was performed on all patients at each of their chemotherapy treatment cycles. For all, except three patients, this included eight images at each of their eight cycles, occurring once every three weeks. Of the remaining three

patients, one individual had succumbed to the disease after cycle two, therefore was only imaged twice and two others received six cycles of treatment, resulting in six images. It is important to note that during the imaging of some patients, US system hardware problems prevented a properly imaged tumor to be stored and therefore in some cases, eight images were not used for measure.

At each visit, 3D US imaging was performed in the clinic, after the medical oncologist had examined the patient and obtained a clinical measure. 3D US imaging is a non-invasive and safe procedure that is completed in just over 5 minutes. The 3D US scanning system used was developed in Dr. Fenster's laboratory, and makes use of a tilt scanning mechanism that allows the transducer to be mechanically tilted in an angular fashion and constant rate over the patient's skin, in order to image the entire tumor structure. To acquire the tumor image, a hand-held scanning motor attachment (developed by Dr. Fenster) holds the transducer, which is placed firmly over the patient's breast tumor. This scanning device rotates the transducer and software simultaneously, acquiring a series of 2D images at regular angular intervals (Fenster *et al.*, 2001). 3D US images were reconstructed using software developed by Dr. Fenster's laboratory.

Once the 3D images were obtained, tumor volumes were measured. Using the 3D segmentation software, manual segmentation was performed by a certified ultrasound technician at St. Joseph's Health Centre, London, Ontario, Canada. The 3D US image is sliced into a series of uniformly spaced, parallel 2D images, from which measurements are taken and used to accurately estimate the tumor volume. Both the number of slices (10-30) and slice increments (0.5-2.0mm) taken for the segmentation were determined by

the overall tumor size. The software then tallied up the 2D conformations into a 3D volume.

All patients' tumor volumes were compared at baseline, based on their diagnostic imaging estimates of tumor volume, with those throughout treatment (using 3D US calculations), to determine volume change over the course of therapy. These volumes were also compared with estimated spherical volumes from the clinical breast examination and final surgical pathology for all patients. In addition, serial *in vivo* 3D US tumor volumes were compared with serial plasma OPN values, to evaluate the correlation between the two methods over treatment.

2.6 Statistical analysis

OPN and tumor volume levels are expressed as medians, with upper and lower quartiles. Repeated measures analysis of variance were used to compare OPN and tumor volume levels across cycles of treatment, with Tukey's multiple comparisons test used to make pair-wise comparisons between cycles. An orthogonal contrast was used to compare the mean OPN levels of cycles 1 through 6 with the mean OPN levels of cycles 7 and 8. Between cycle comparisons of elevated OPN levels (>123 ng/ml) were made using McNemar's chi-square test. The relationship between OPN levels and response was evaluated using a Wilcoxon two-sample test. The relationship between elevated OPN and response was evaluated using Fisher's exact test.

Associations between OPN and tumor volume were evaluated using Pearson correlation coefficients. Correlation coefficients greater than 0.50 were considered to be good. Probability values ≤ 0.05 were regarded as being statistically significant.

Chapter 3: Results

3.1 OPN blood levels of individual patients, over the course of treatment

Fig. 3.1 shows the changes in plasma OPN levels in ng/mL for each of the nineteen patients over the course of neoadjuvant chemotherapy. In addition, final response to neoadjuvant therapy is denoted for each patient by CR (complete response), PR (partial response), NR (no response) or PD (progressive disease).

Patient 12 had only two OPN plasma samples measured as a result of this patient's death during treatment. This patient was the only male enrolled in the study. A baseline OPN level of 111ng/mL was measured by ELISA, which, at cycle two was elevated to 395ng/mL prior to his death. This large increase raised the OPN level above the previously reported value for the upper limit of normal, 123ng/mL (Bramwell *et al.*, 2006). This patient had an initial diagnostic tumor volume of $65cm^3$, however the final tumor volume was not known, as no pathology was completed after death. Response to neoadjuvant chemotherapy was determined to be PD, as a result of his death before treatment was completed.

Only two of the nineteen patients, patient 10 and 15, had a CR to neoadjuvant chemotherapy. These individuals had no evidence of disease at final pathology. Both patients had baseline OPN levels well below the upper limit of normal, at 90ng/mL and 66ng/mL, respectively. Over the eight courses of neoadjuvant treatment, patient 10's plasma OPN levels remained below 123ng/mL for the first five cycles and then increased from cycle six until cycle eight, to a final high of 202ng/mL. Similarly, patient 15's plasma OPN levels also remained below 123ng/mL for the first six cycles, only being elevated to 101ng/mL.

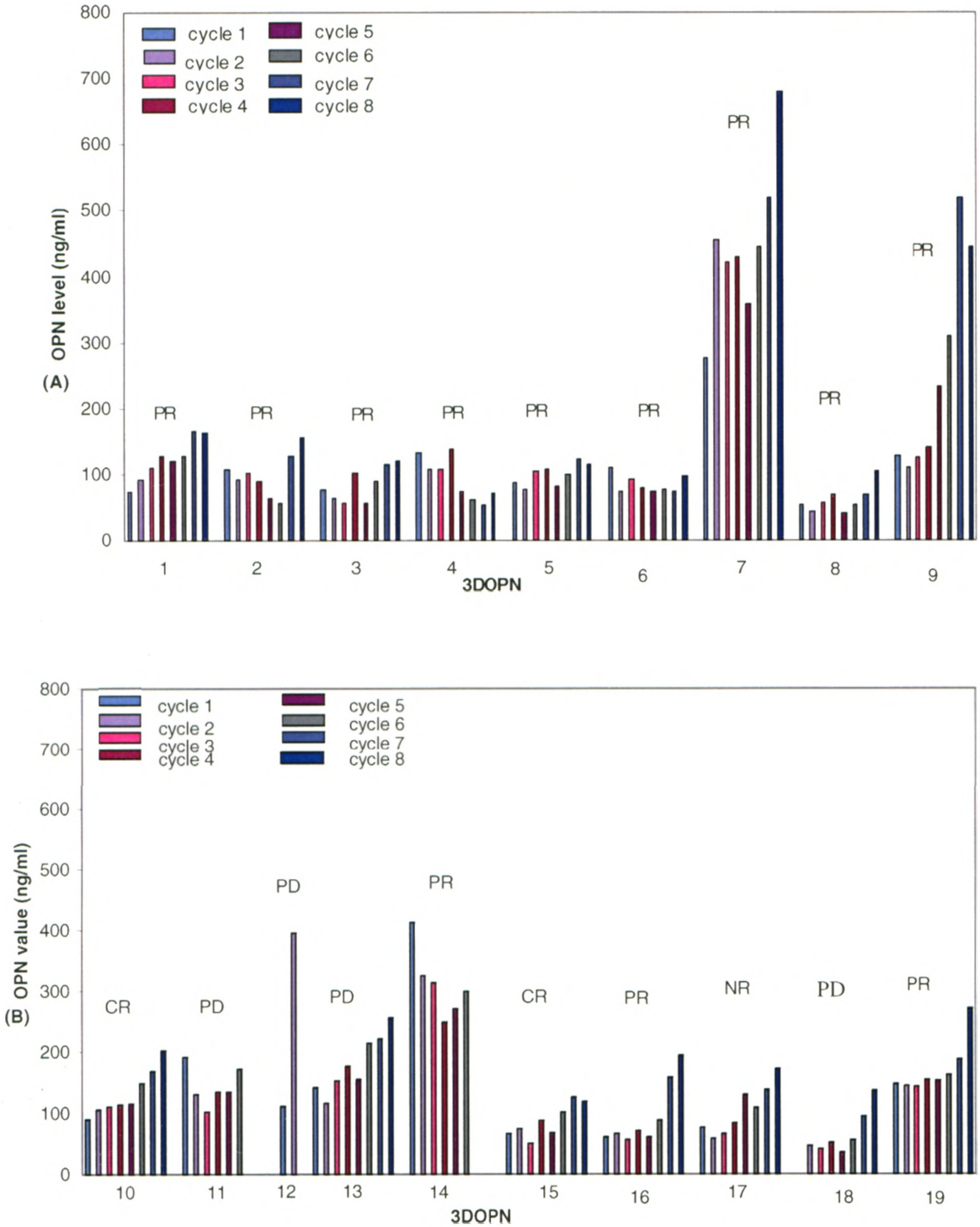

Figure 3.1 Plasma OPN levels (ng/mL) over the eight cycles of neoadjuvant therapy for patients 1-9 (A) and 10-19 (B) with final response to treatment. At every treatment cycle plasma OPN samples were measured three times by ELISA and median OPN level in ng/mL is shown. Final patient response is denoted as complete response (CR), partial response (PR), no response (NR) or progressive disease (PD).

At cycle seven, OPN levels increased to a high of 126ng/mL, but then decreased to 119ng/mL at cycle eight.

Patient 17 was the only individual who had NR to treatment. Baseline OPN levels were found to be in the normal range, at 76ng/mL and remained low until cycle five. At this time plasma OPN levels were found to increase to 130ng/mL. At cycle six, levels were found to decrease, only to increase again to an elevated high of 172ng/mL at cycle 8.

Two interesting cases can be seen with patients 7 and 14, both of whom had PR. Baseline OPN plasma levels were elevated above the normal limit in both cases, with patient 7 having a value of 276ng/mL and patient 14 a level of 413ng/mL. In the case of patient 7, OPN levels remained elevated above123ng/mL throughout all of the treatments and continued to rise, until a high of 680ng/mL at cycle 8. For patient 14, OPN levels all remained elevated above the 123ng/mL level, with a final measure of 299ng/mL. It was interesting to note that patient 14 had bilateral tumors, with a smaller tumor being located in the right breast, and the left breast's larger tumor being used for imaging in the study. In addition, after commencement into the study, multiple sites of metastasis were discovered, involving the axillary and appendicular skeleton. Although both patients had a PR, with a diagnosis of metastasis, patient 14 has a poor prognosis.

Patients 1-6, 8 and 16 all had a PR to neoadjuvant therapy. These individuals all had baseline plasma OPN levels below 123ng/mL and remained low until later into the treatment cycles. Patient 9 and 19 also had a PR, however, they initially had elevated baseline OPN levels, measured at 128ng/ml and 147ng/ml, respectively. These levels remained high throughout the completion of treatment, reaching 444ng/mL for patient 9,

and 271ng/mL for patient 19. Interestingly these individuals had a 99% response to treatment with very little residual disease remaining at pathology.

Patients 11, 13 and 18 were all cases of PD while on treatment; however, there was lack of a trend in plasma OPN levels observed. Both patient 11 and 13 had elevated OPN measures at baseline, which remained elevated throughout the entire treatment cycle, except for one cycle early on. Patient 11 was discovered to have metastasis after her enrollment and commencement of the study. Patient 18 however, had a baseline OPN level below 123ng/mL, which although remaining below this cut off for most subsequent samples, was found to increase, with the final OPN measure elevated to 137ng/mL.

3.2 Changes in group plasma OPN levels throughout neoadjuvant therapy

Changes in plasma OPN levels for the entire group throughout the eight cycles of chemotherapy was examined, with results displayed in Figure 3.2. Median OPN levels for all the patients for cycles one through eight of neoadjuvant therapy is shown. Based on log transformation, there was found to be a significant difference in the mean OPN levels across the eight cycles of treatment ($p < 0.0001$). The OPN changes between successive cycles show no significant difference between levels at baseline through to cycle six. At these early cycles, median OPN levels remain between 91ng/mL and 112ng/mL. Median OPN level at cycle seven increased to 133ng/mL (with a lower quartile of 105ng/mL and a upper quartile of 178ng/mL) and further increased to 160ng/mL (lower quartile 117ng/mL and upper quartile 229ng/mL) at cycle eight. The differences between OPN levels at the early cycles (one–six) compared to the late cycles (seven and eight), shows a significant difference (increasing in late cycles) in plasma OPN levels ($P<0.0001$).

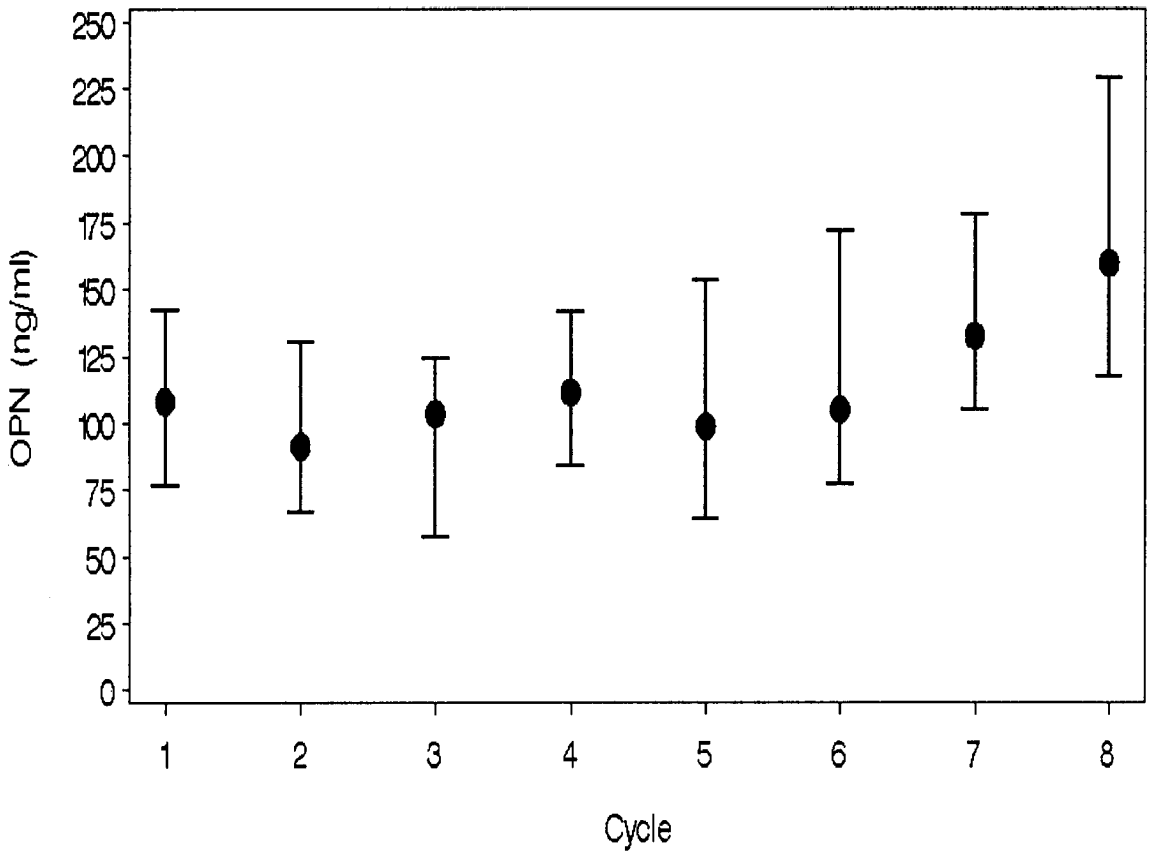

Figure 3.2 Changes in group OPN levels throughout the 8 cycles of neoadjuvant treatment. Group median OPN levels in ng/mL as measured by ELISA. OPN levels at cycle 1-6 are significantly statistically different from cycles 7 and 8 with $p<0.0001$. Upper and lower quartiles are shown at each point.

3.3 Elevated plasma OPN levels at baseline and over the course of neoadjuvant therapy

At baseline, 7 out of 18, or 39% of LABC patients had median plasma OPN levels elevated above 123ng/mL and 61% (11 out of 18) of the patients had plasma OPN levels below this upper limit of normal (Figure 3.3). Figure 3.4 shows the percent of patients with elevated OPN plasma levels at each treatment cycle over the course of neoadjuvant chemotherapy. Throughout the treatment cycles, there appears to be a trend in the percent of patients with elevated OPN plasma levels increasing, after an initial decrease at cycle two (26%) and cycle three (28%) (Figure 3.4). Nearing the completion of neoadjuvant chemotherapy, 69% of patients (11 out of 16) had a median OPN level that was elevated above 123ng/mL at cycle seven and 63% (10 out of 16) had elevated OPN levels at cycle eight. Pair-wise comparisons were done to determine if this increase in the percent of patients with elevated OPN levels at late cycles differed from the early cycles. These comparisons showed that there were statistically significant differences in some of the early cycles (2, 3 and 5) versus late cycles (7 and 8) (Figure 3.4).

3.4 LABC patient response to neoadjuvant therapy

After neoadjuvant chemotherapy was completed, the response of each patient was determined by the final pathology of the residual tumor after surgery. Patients were categorized as complete response (CR), partial response (PR), no response (NR) or progressive disease (PD), based on the presence and size of their residual invasive tumor after surgery. Figure 3.5 shows the percent of LABC patients in each category of response.

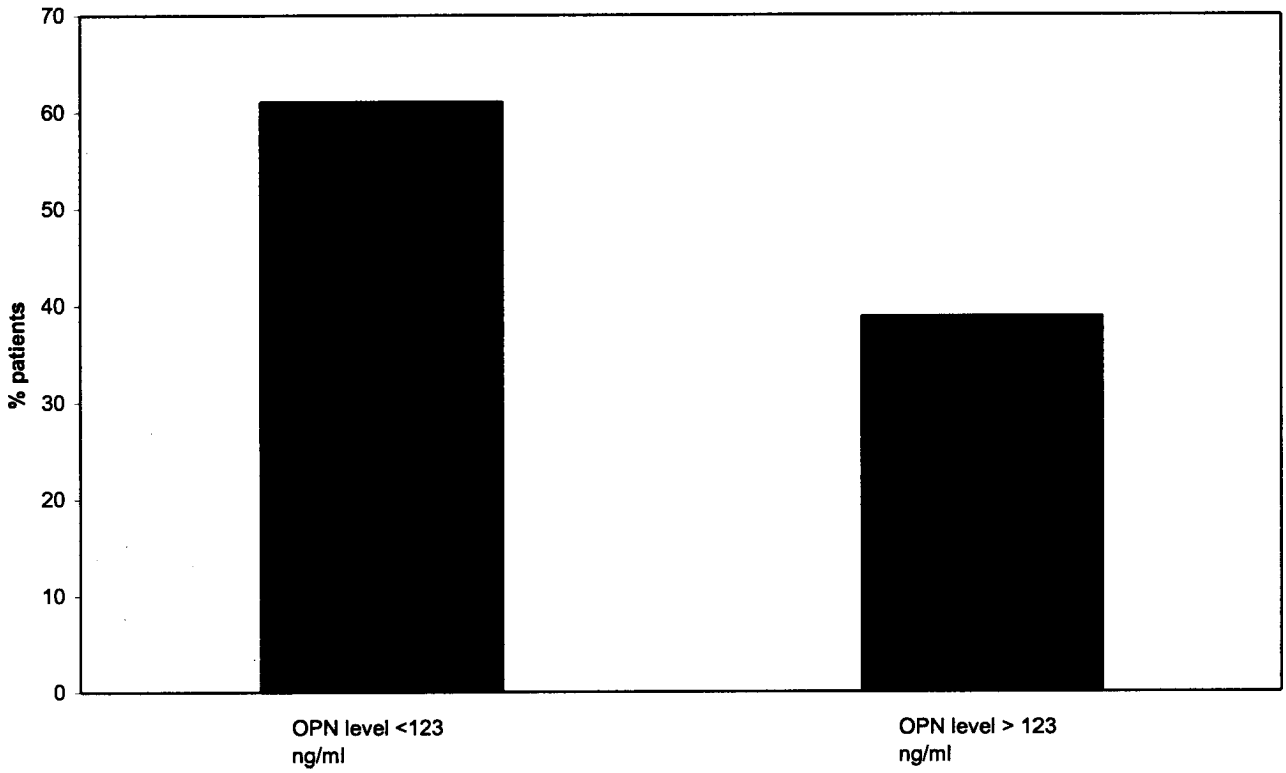

Figure 3.3 Percent of patients with OPN levels (ng/mL) below (light blue bars) and above (dark blue bars) the upper limit of normal, 123 ng/mL, at baseline.

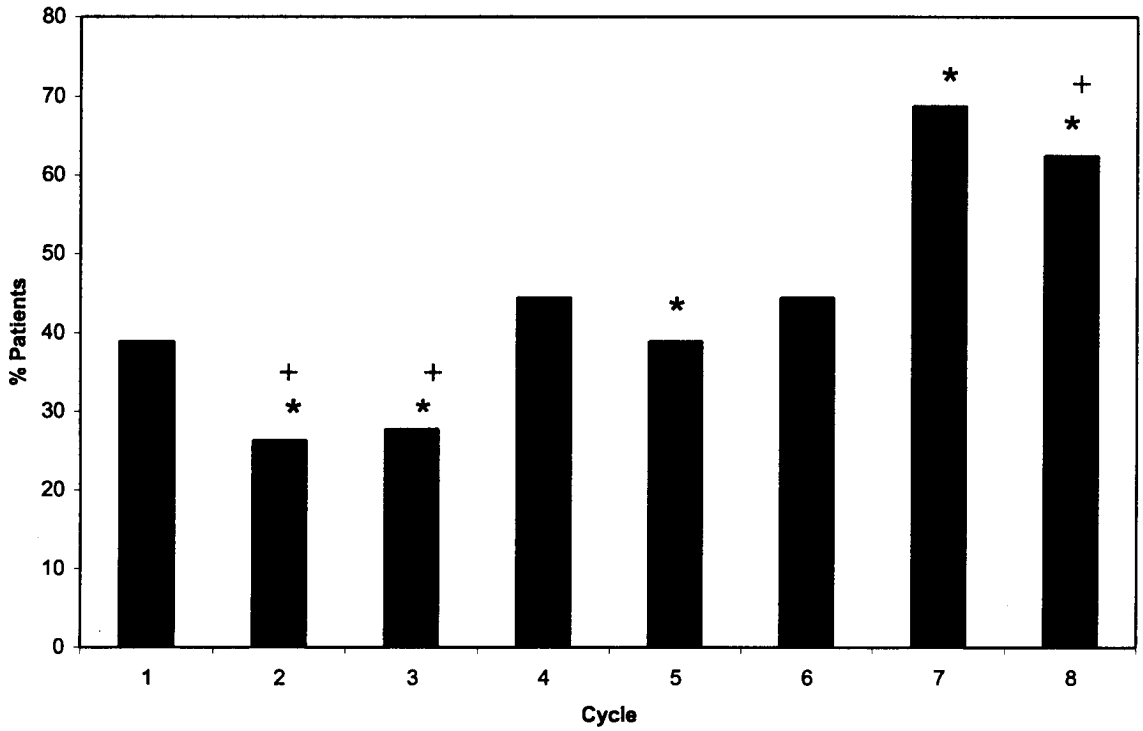

Figure 3.4 Percent of LABC patients with OPN levels elevated above the upper limit of normal (123ng/mL) at each treatment cycle over the course of neoadjuvant therapy. Pair-wise comparisons were completed between all cycles to determine if there was a significant difference, as indicated by asterisks and cross (P value at least less then 0.05). Percent of patients with elevated levels at cycle 2 (p= 0.004), cycle 3 (p= 0.02) and cycle 5 (p=0.03) was statistically lower than those at cycle 7. The percent of patients with OPN levels elevated at cycle 2 (p=0.008) and at cycle 3 (p=0.03) was statistically different than those at cycle 8.

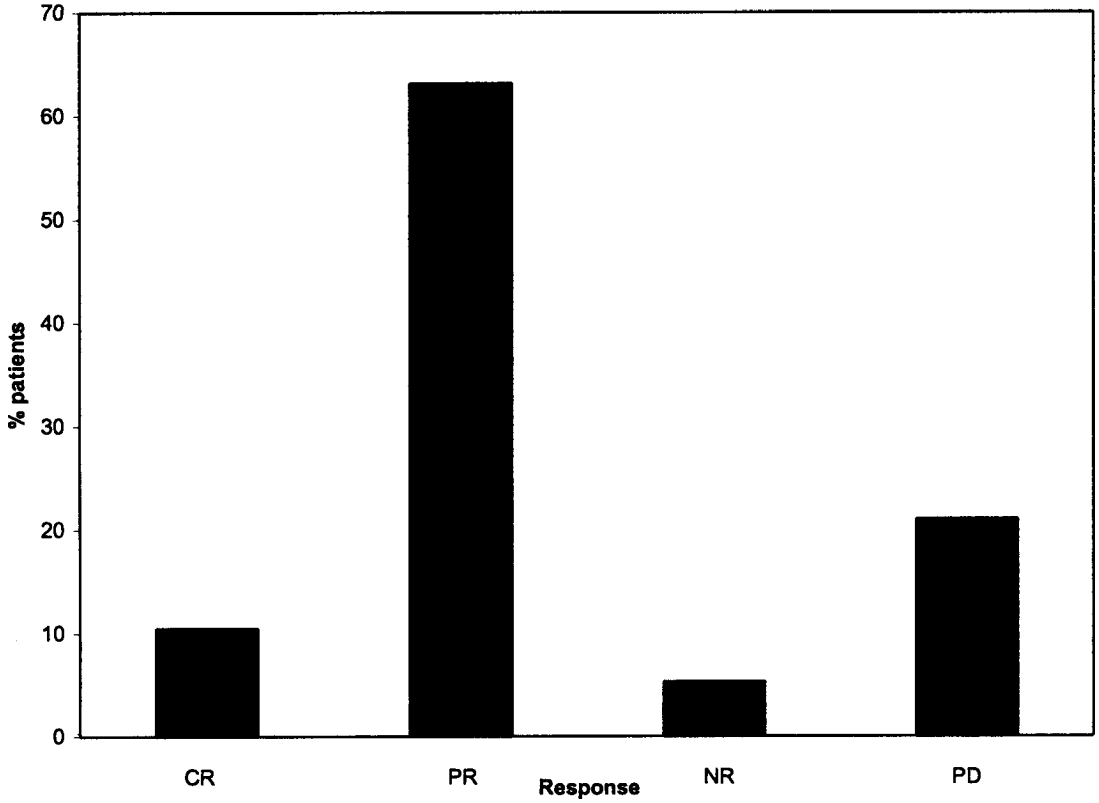

Figure 3.5 LABC patient response to neoadjuvant therapy. Shown is the percent of patients that had a complete response (CR), partial response (PR), no response (NR) or progressive disease (PD).

This figure shows that the majority of LABC patients had a partial response to neoadjuvant therapy, with 63% (12 out of 19) of patients falling into this category. Of the patients, two (11%) were found to have a complete response, four (21%) had progression of disease and only one individual (5%) had no response to treatment.

3.5 Plasma OPN levels and the relationship with response to neoadjuvant chemotherapy

Plasma OPN levels were compared with the final response to neoadjuvant chemotherapy for all LABC patients. Figure 3.6 shows the final response that patients with elevated baseline OPN levels had to treatment. Of the fourteen individuals who had a complete or partial response to treatment, five of them or 36%, had baseline OPN levels that were elevated above 123ng/ml (the upper limit of normal) (Figure 3.6). Of the four patients having no response or progressive disease, 50%, or two individuals had levels above 123ng/ml (Figure 3.6). (Note patient 18 was excluded as no baseline OPN levels were obtained). Although there seems to be a trend for those with elevated baseline OPN levels to have progressive disease or no response, this difference was not statistically significant by Fisher's exact two-tailed test within the power of this pilot study. Breaking this down further into individual categories, of the patients that had a progressive disease, 67% (2 out of 3) had baseline plasma OPN levels above normal and 42% or five of the twelve patients with a partial response had elevated levels. Interestingly, of the patients with elevated OPN levels, none had a complete response. However, this requires further investigation as only two individuals out of all nineteen patients in the study had a complete response.

Median baseline plasma OPN level was compared to the final response for all the patients. As shown in Figure 3.7, patients exhibiting a complete or partial response had a median OPN level of 99ng/ml (lower quartile 74ng/ml; upper quartile 134ng/ml), which was lower than the patients that had disease progression or no response, at 127ng/ml, (lower quartile 94ng/ml; upper quartile 167ng/ml). This trend of a lower baseline OPN level associated with a better response to treatment was not statistically different (p-value of 0.43) within the power of this pilot study, but may be of clinical importance and is worthy of further investigation. Table 3.1 shows the final tumor volume each patient had following surgery after neoadjuvant therapy compared to baseline OPN levels.

Finally, the final median plasma OPN level was compared to final response. OPN levels for non-responders or for patients with progressive disease was found to be 172ng/ml (lower quartile 137ng/ml, upper quartile 256ng/ml), which is higher than the median level, 157ng/ml (lower quartile 116ng/ml, upper quartile 202ng/ml), for complete and partial responders (Figure 3.8). Again this difference indicates a trend but has no statistically significant difference ($p= 0.59$).

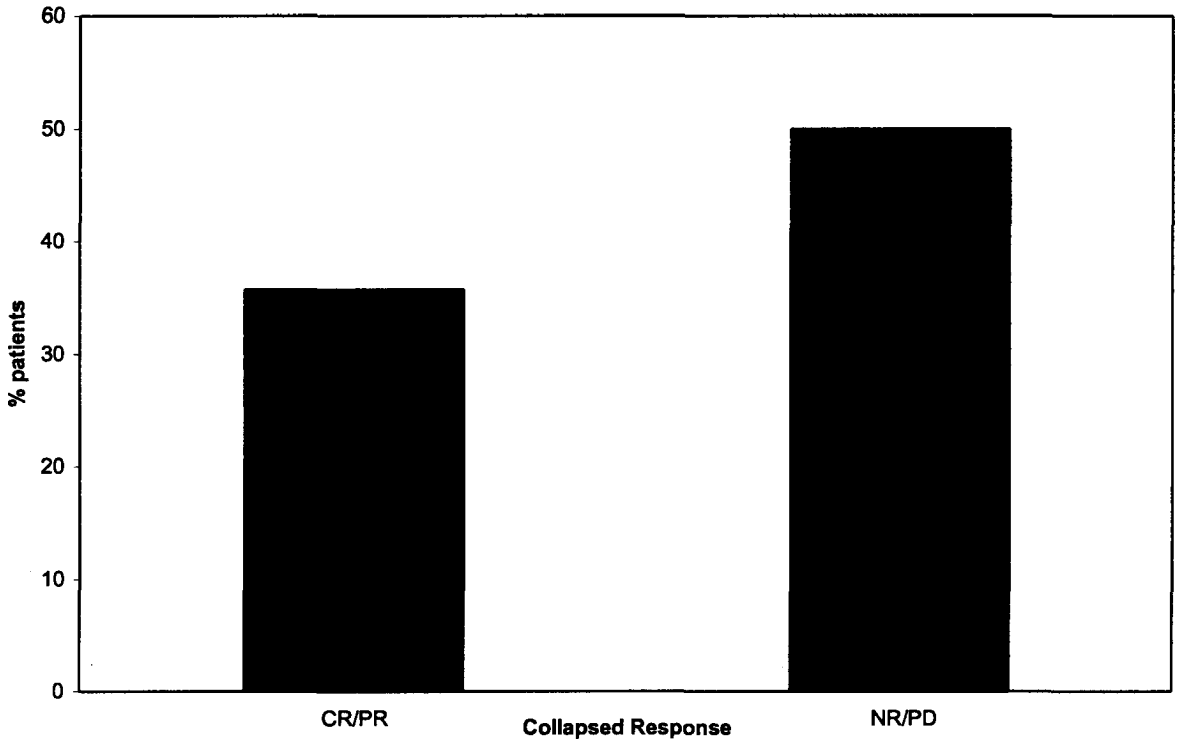

Figure 3.6 Response to neoadjuvant treatment for patients with elevated (>123 ng/ml) OPN levels at baseline. For patients with elevated baseline OPN levels (N=7) there was a trend towards a higher percent of patients with no response or progressive disease vs. complete or partial response, although this was not found to be significantly different statistically, within the power of this pilot study (P> 0.999).

Patient	Plasma OPN at baseline (ng/mL)	Final pathological tumor volume (cm^3)
1	74	14.13
2	107	0.52
3	77	50.94
4	134	0.065
5	86	0.471
6	109	220.78
7	276	1.95
8	55	1.44
9	129	0.001
10	90	0
11	192	904.32
12	111	-
13	142	179.5
14	412	10.3
15	66	0
16	61	0.203
17	76	1022
18	-	12763.57
19	147	0.0005

Table 3.1 **Baseline plasma OPN levels (ng/mL) and final tumor volume for all nineteen LABC patients.**

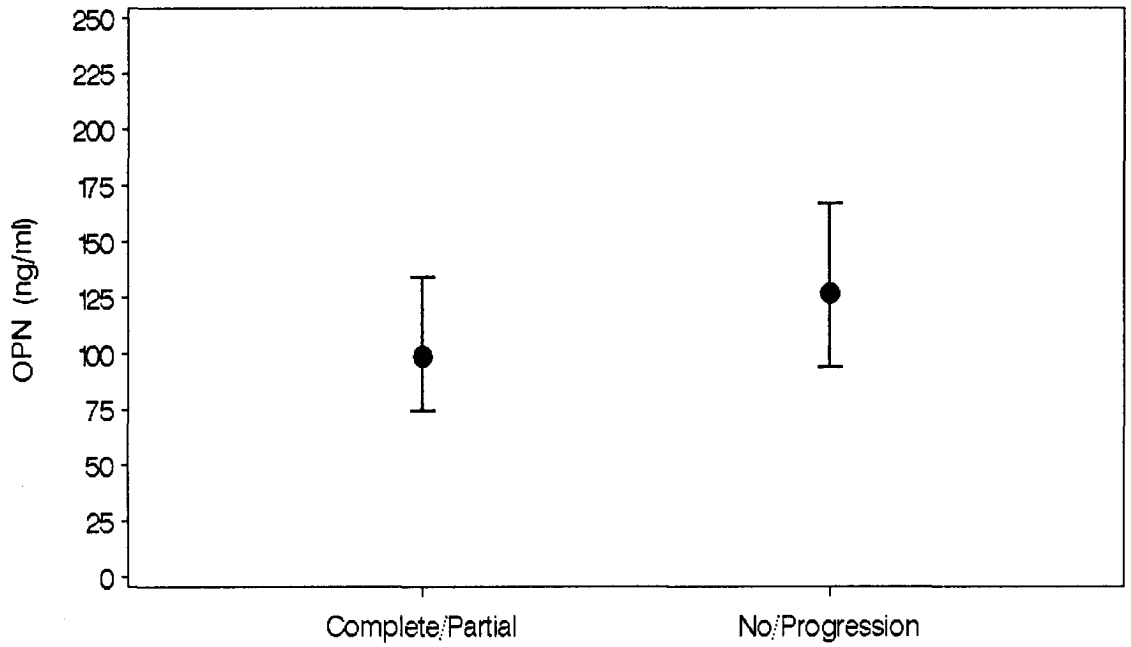

Figure 3.7 Median baseline OPN level compared to final response to neoadjuvant therapy for all nineteen patients. There was no statistical difference between the two groups (p= 0.43). Upper and lower quartiles shown for each point.

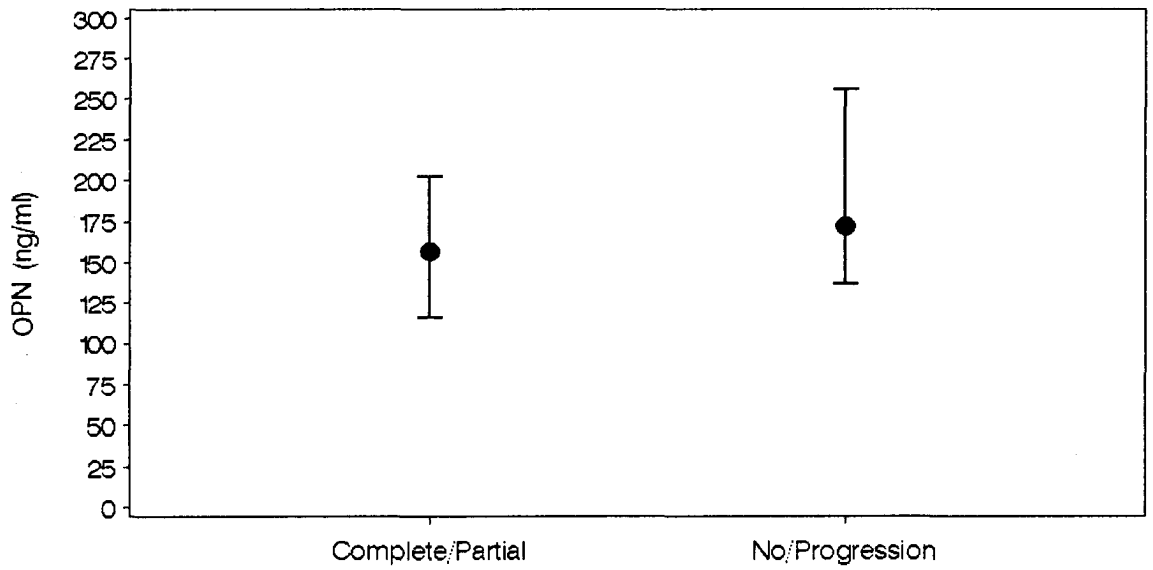

Figure 3.8 Final median OPN levels compared to final response for all nineteen patients. There was no statistical difference between the two groups, p=0.59. Upper and lower quartiles shown for each point.

3.6 Patient tumor volumes

Throughout the course of neoadjuvant therapy, patients had tumor volumes measured clinically, as well as with 3D US. Clinical estimates of tumor volume for all nineteen patients were obtained by the medical oncologist. Patients 1, 6, 13 and 14 had tumors that were estimated to be unpalpable after treatment with neoadjuvant therapy, although final pathology determined that a large tumor still remained. Patient 2 had a final clinical estimation of tumor volume at cycle eight of 0.52 cm^3, which also was found to be the final measure of the residual tumor on pathology. Similarly, patient 4 had an accurate clinical estimation. Although her tumor was considered to be unpalpable, final pathology showed a residual mass of 0.065 cm^3, which was similar to the estimation at cycle 5. Patients 7 and 9 had large clinical estimations of their tumors at cycle 8, however on final pathology, the residual tumor was much smaller than the estimate. Estimations of tumor volume for patients 3, 11, 17 and 18 were all underestimated clinically vs final pathology. It is important to note that for all nineteen patients there was a lack of an estimation at various cycles over the course of treatment. The reason for the lack of an estimate was often due to the tumor being unpalpable, no definite margins found, presence of a very large tumor volume or simply out of error.

In addition, all patients had tumor volume measured using the 3D US at each cycle. Tumor volume changes (in cm^3) were measured by 3D US over the eight cycles of neoadjuvant therapy. Patients 2, 4, 5, 7, 8, 9, 14, 16 and 19 all had a very small residual tumor at final pathology, however volume was overestimated with the use of the 3D US. Patients 10 and 15 had a complete response to treatment and therefore at final pathology there was no evidence of residual disease, yet with the use of 3D US there was found to

be a 2.37 cm^3 and a 4.7 cm^3 tumor volume, respectively. Underestimates with 3D US imaging were seen with patients 3, 6, 11, 13, 17 and 18. These individuals had a small tumor volume obtained by imaging with 3D US at final treatment cycle however, after pathology, it was determined that these individuals still had a large residual tumor present. Lack of a 3D volume in patients 2, 8, 11 and 18 at different cycles was due to US hardware problems which prevented 3D US from being obtained.

3.7 Changes in 3D tumor volume for the whole group over the course of treatment

Median tumor volume changes for the patient population were examined over the course of eight cycles of neoadjuvant therapy. Median tumor volumes, in cm^3, are shown in Figure 3.9. Median group tumor volume at baseline was found to be 36.15 cm^3 (lower quartile 27.57 cm^3, upper quartile 49.00cm^3). Over the cycles, this volume is shown to decrease, with a final tumor volume at cycle eight of 8.76 cm^3 (lower and upper quartile, 4.70cm^3 and 17.01cm^3). There was a trend of decreasing tumor volume over the eight cycles, on average for all patients, although the differences were not statistically significant within the power of the present study ($p= 0.247$).

3.8 3D US volume and relationship with response to neoadjuvant therapy

Median tumor volumes from 3D US at baseline and final treatment cycle were compared with final response to neoadjuvant treatment. For individuals having a complete or partial response to neoadjuvant therapy, their median tumor volume, as measured by 3D US, was found to be 36.11cm^3 (lower quartile 27.13cm^3, upper quartile *49.00cm^3), at baseline (Figure 3.10).*

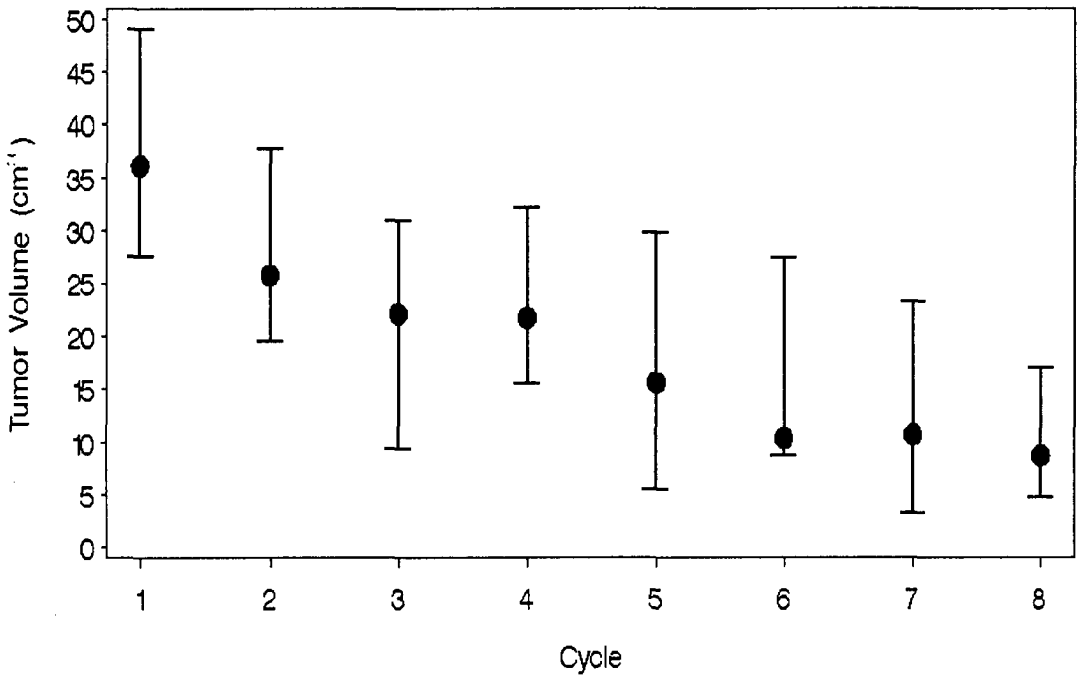

Figure 3.9 Changes in tumor volume as measured by 3D US over the eight cycles of neoadjuvant therapy for all 19 patients. Although there appears to be a trend of decreasing tumor volume, differences are not statistically significant, p= 0.247. Upper and lower quartiles are shown with bars at each data point.

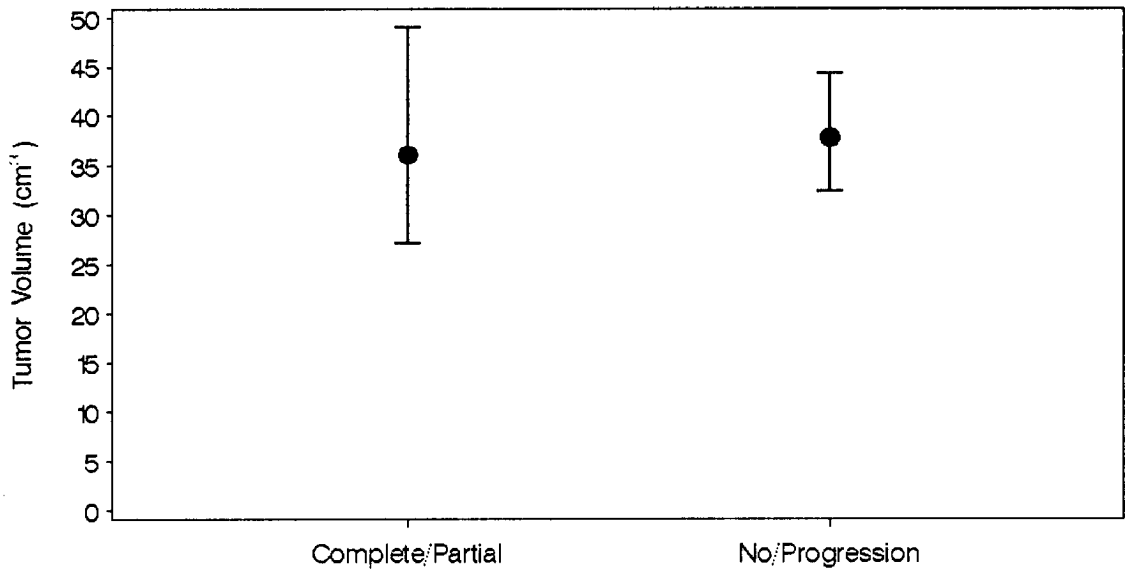

Figure 3.10 3D US tumor volume at baseline for patients having a complete or partial response, compared to tumor volume for patients having no response or progressive disease. There was no statistically significant difference, p=0.677. Upper and lower quartiles represented as bars at each point.

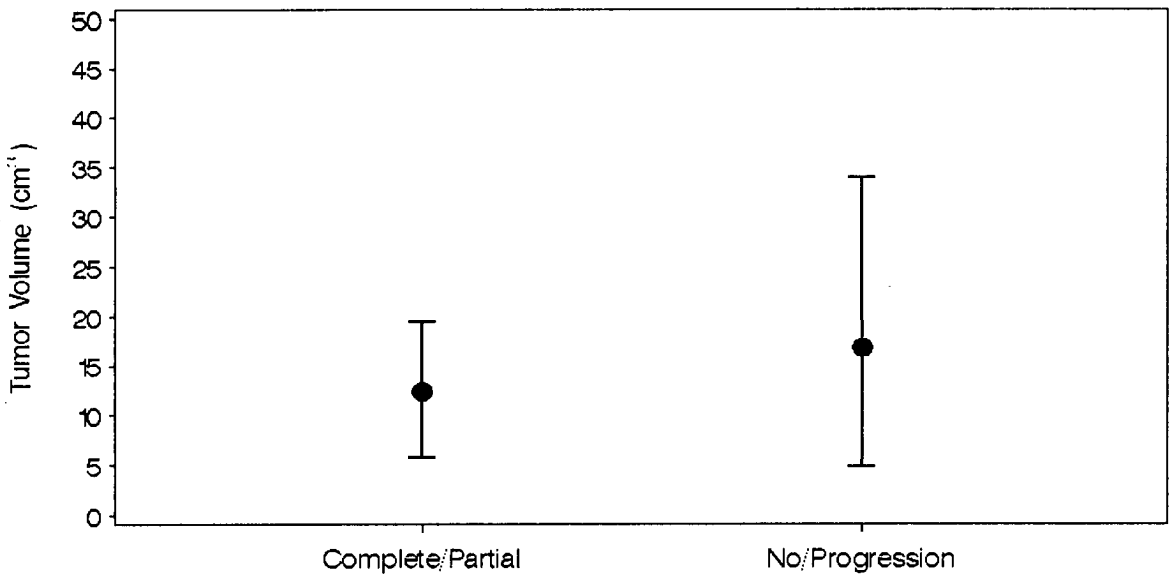

Figure 3.11 Final tumor volume by 3D US for patients having complete or partial response, compared to patients having no response or progressive disease. There was no statistically significant difference, p= 0.791. Upper and lower quartiles are represented as bars at each data point.

This volume appears to be smaller than the tumor volume for individuals with no response or progressive disease, 37.80 cm^3 (lower and upper quartiles, 32.30cm^3 and 44.26cm^3), although not significantly different statistically (p=0.677).

Final tumor volume from 3D US was also compared with the final response to treatment. A trend indicating that smaller tumor volumes at the final chemotherapy cycle indicate a more favorable response was seen, although this was not significant (p=0.791). For patients who eventually were found to have a complete or partial response to treatment, median tumor volume was 12.38 cm^3 (lower & upper quartiles, 5.78cm^3 & 19.44cm^3) at cycle eight, compared to patients who had no response or progressive disease, who showed a median volume of 16.81 cm^3 (lower & upper quartiles, 4.76cm^3 & 33.96cm^3) (Figure 3.11).

3.9 Correlation of diagnostic tumor volume with baseline clinical and 3D US volume

The relationship between the diagnostic tumor volumes (as routinely assessed using mammography, ultrasound and MRI) and the clinical tumor volume (as routinely assessed by the oncologist), was examined. Table 3.2 shows the tumor volumes at baseline as well as final from diagnostic imaging, clinical estimation and 3D US which were used in correlations. Figure 3.12 shows the correlation between diagnostic tumor volume and baseline clinical tumor volume estimation. Only fourteen out of the nineteen patients' baseline measures were available for use, due to five patients not having their primary tumor clinically assessed at baseline. Figure 3.12 shows that for these fourteen patients there was a good statistical correlation between diagnostic tumor volume and clinical tumor volume estimation ($r= 0.525$; $p= 0.054$).

Patient	Diagnostic Volume (cm^3)	Clinical baseline estimate (cm^3)	3D US baseline volume (cm^3)	Clinical estimate final volume (cm^3)	3D US volume final (cm^3)
1	54.33	54.33	28.2	-	8.76
2	0.9	22.44	7.8	0.52	3.79
3	113.04	113.04	45	-	-
4	33.49	113.04	61.81	-	17.01
5	28.72	267.95	49	-	5.78
6	321.39	267.95	53.78	-	11.88
7	523.33	-	53.07	179.5	8.57
8	143.72	65.42	9.28	4.19	13.55
9	8.18	179.5	6.46	14.13	12.88
10	65.94	696.56	36.06	0.06	2.37
11	7.23	-	32.3	523.33	26.27
12	65.42	-	50.49	267.95	20.96
13	113.04	-	37.8	-	7.34
14	87.07	381.51	27.52	-	25.65
15	47.69	33.49	27.13	-	4.7
16	267.95	533.33	36.15	-	22.67
17	904.32	696.56	28.92	-	41.64
18	11.49	523.33	44.26	-	2.17
19	299.24	-	38.82	-	32.5

Table 3.2 Baseline and final volumes (cm^3) from diagnostic imaging, clinical estimation and 3D US imaging for all LABC patients.

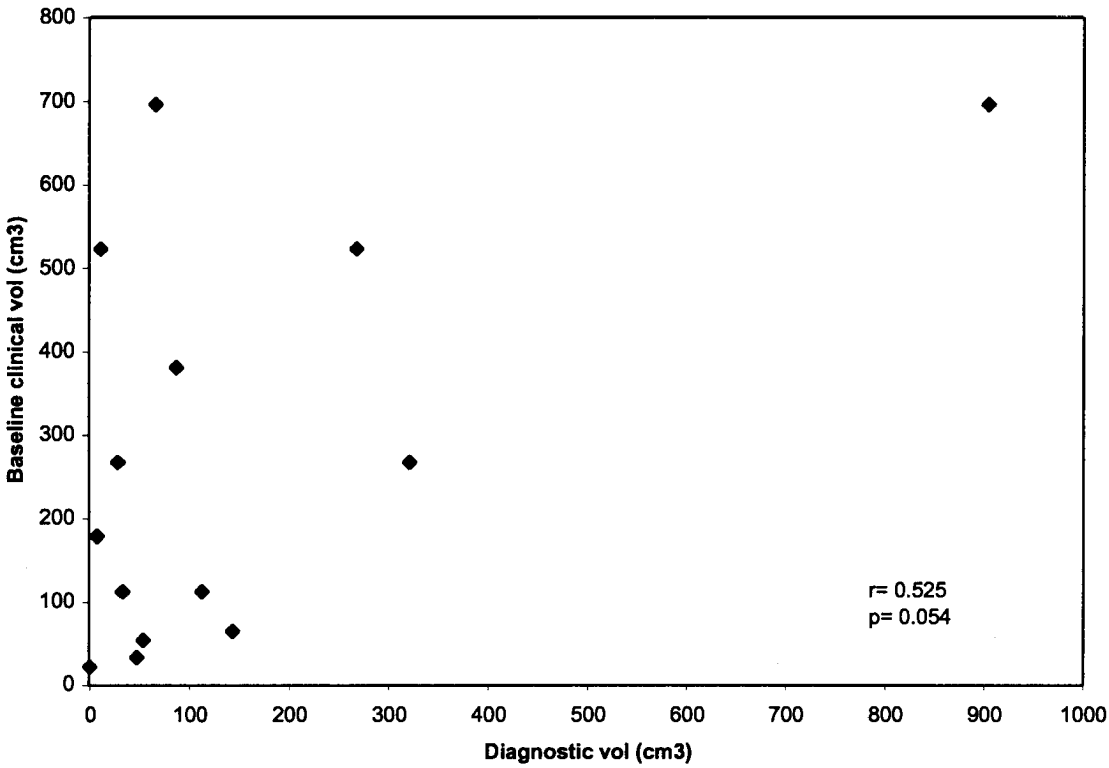

Figure 3.12 Correlation of diagnostic tumor volume with clinical baseline tumor volume estimation for 14 LABC patients receiving neoadjuvant therapy. r= 0.525; p= 0.054.

Correlation between diagnostic volume and baseline 3D US volume was also examined for all nineteen patients. Figure 3.13 shows that there was no correlation statistically between diagnostic tumor volume and volume obtained at baseline using 3D US, with r= 0.133 and p= 0.588.

Lastly, the relationship between the clinical estimation of tumor volume and the tumor volume measure obtained by 3D US was assessed. There was found to be no correlation statistically at baseline between these two methods of obtaining tumor volume for the fourteen patients who had a baseline clinical measure(r= 0.221; p= 0.449) (Figure 3.14).

Correlation between clinical estimation of tumor volume by the medical oncologist and measurement of tumor volume obtained from 3D US were also examined over the remaining six cycles of neoadjuvant therapy. For patients with both measurements at the six treatment cycles, there was found to be no statistically significant correlation between clinical estimate of tumor volume and tumor volume obtained from 3D US imaging. Figure 3.15 shows this relationship at cycle 3 of treatment. Although r=0.616, this correlation was not statistical (p=0.193).

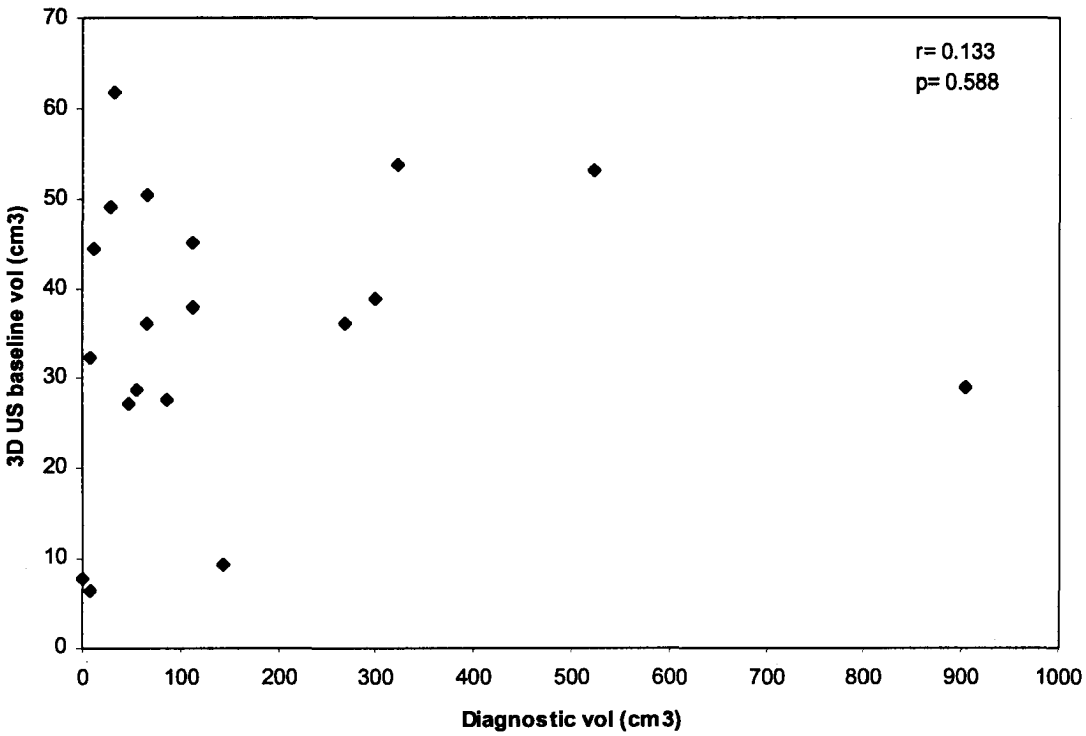

Figure 3.13 Correlation of diagnostic tumor volume with baseline 3D US tumor volume for all nineteen LABC patients receiving neoadjuvant therapy. r= 0.133; p= 0.588.

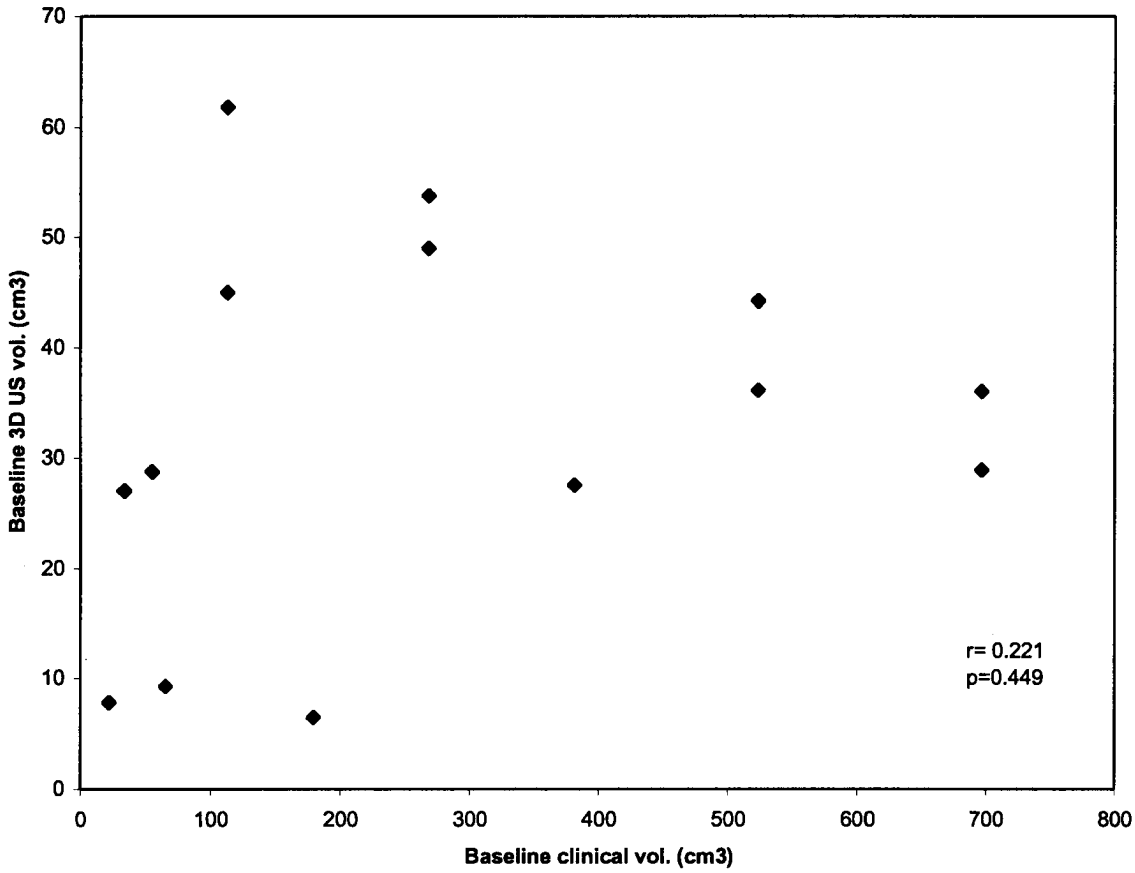

Figure 3.14 Correlation of tumor volume at baseline obtained from clinical exam and 3D US imaging of fourteen LABC patients receiving neoadjuvant therapy. r= 0.221; p= 0.449.

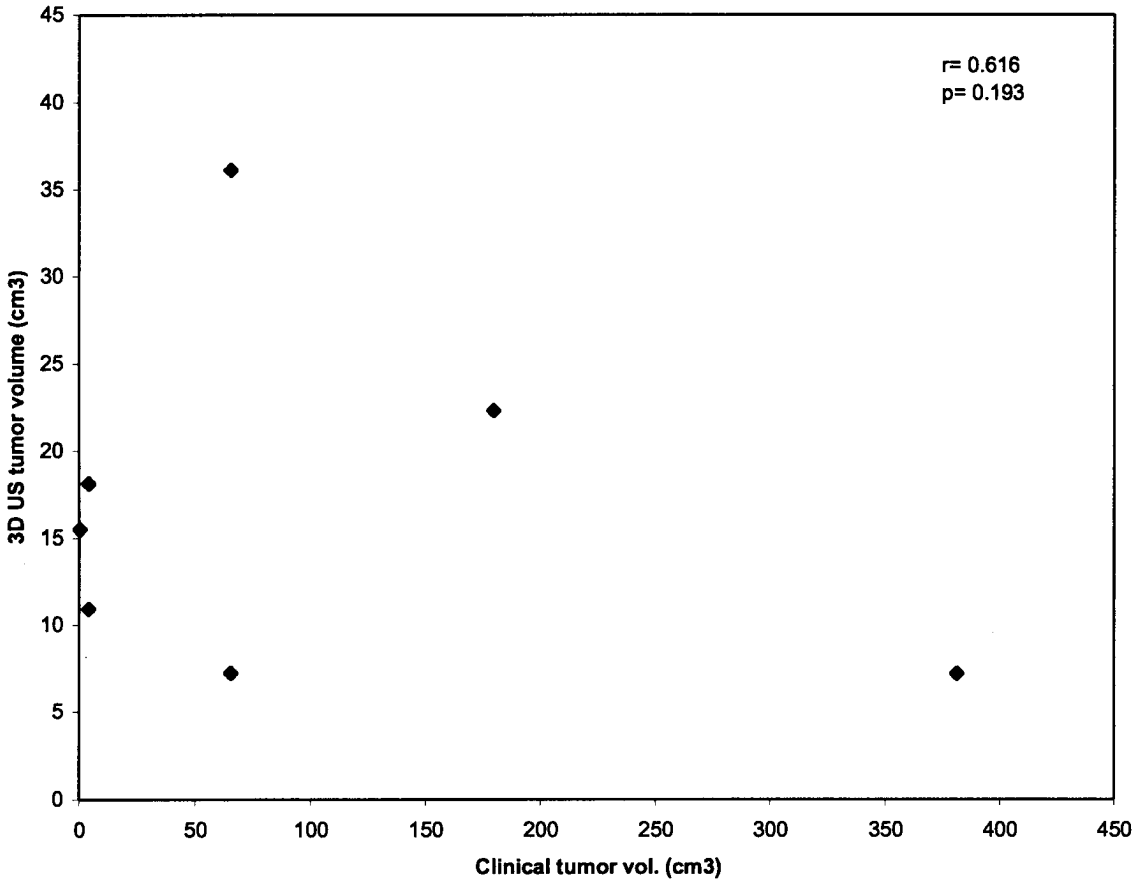

Figure 3.15 Correlation of clinical estimation of tumor volume with tumor volume measured by 3D US at cycle 3 of neoadjuvant treatment, for seven LABC patients. r= 0.616; p= 0.193.

3.10 Correlation between final tumor volume (from pathology) and final clinical and 3D US tumor volume

The relationship between final tumor volumes from pathology (after eight cycles of neoadjuvant chemotherapy) and final clinical estimation of tumor size was examined in Figure 3.16. Seven of the nineteen LABC patients had a clinically palpable tumor remaining at the end of treatment, from which an estimation of size was obtained. Final tumor volume from pathology was found to be strongly correlated statistically with final clinical estimation at cycle 8 for seven patients who had clinically palpable tumors after treatment ($r= 0.943$; $p= 0.005$).

The relationship between final tumor volumes from pathology and final tumor volumes from 3D US at cycle 8 (cycle 6 for patients 11 and 14) was also investigated. For eighteen patients, Figure 3.17 shows that there was no statistically significant positive correlation between final tumor volume from pathology and final 3D US volume ($r= -0.215$; $p= 0.407$).

Finally, the relationship between the two methods of interest, clinical estimation and 3D US volume measurement, was examined with respect to the final tumor volume. For the seven patients with palpable tumors at the final cycle, the clinical estimations of tumor size was found to be statistically correlated with the final tumor volume obtained from the last 3D US measurement, as shown in Figure 3.18 ($r= 0.829$; $p= 0.021$).

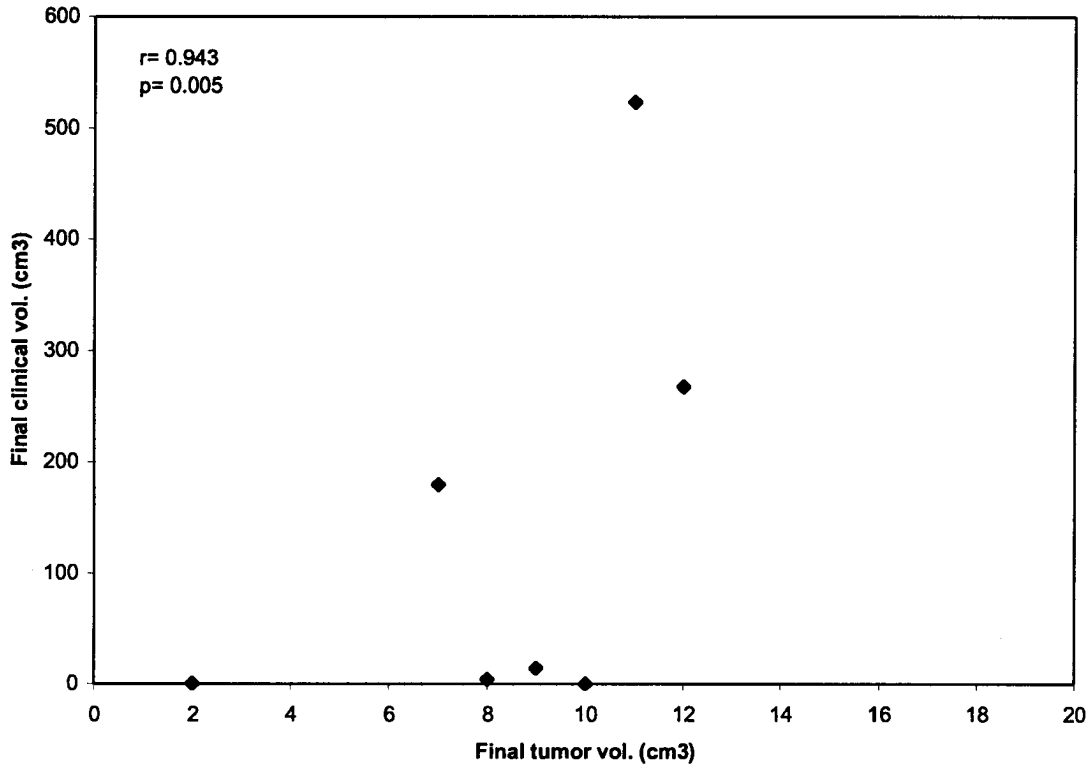

Figure 3.16 Correlation of final tumor volume from pathology with final clinical exam estimation of tumor volume, for seven LABC patients after neoadjuvant treatment. r= 0.943; p= 0.005.

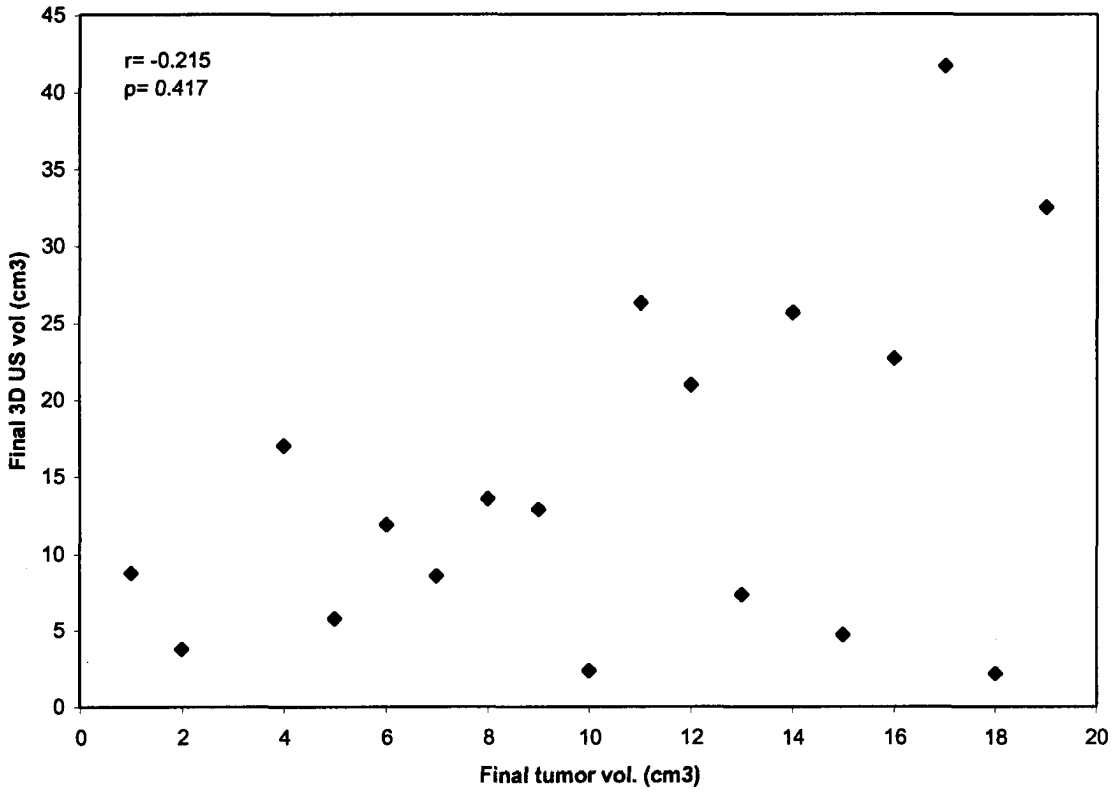

Figure 3.17 Correlation between final tumor volume from pathology and final 3D US tumor volume, for eighteen patients after receiving neoadjuvant chemotherapy for LABC. r= -0.215; p= 0.407.

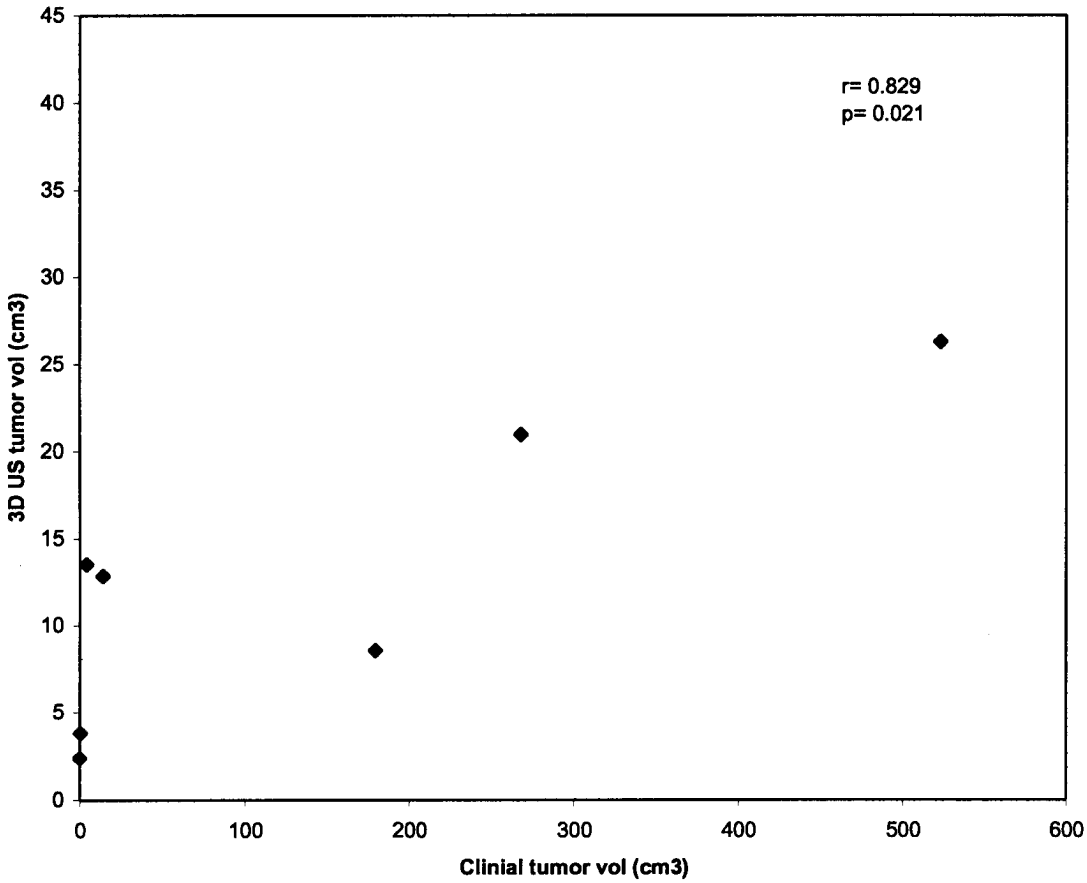

Figure 3.18 Strong correlation of final clinical estimation of tumor volume with final tumor volume measured using 3D US imaging, on seven LABC patients after completing neoadjuvant therapy. r= 0.829; p= 0.021.

3.11 Relationship between OPN and 3D US imaging

The relationship between OPN level and 3D US imaging was then examined for all cycles of neoadjuvant treatment. For all eight cycles of neoadjuvant treatment, there was found to be very little evidence of an association between plasma OPN level and 3D US tumor volume measure. Figure 3.19 shows that at cycle 1, there is no correlation between baseline OPN levels and tumor volumes by 3D US for eighteen patients ($r= 0.099$; $p= 0.696$). At cycle 8, there was found to be no correlation between OPN level and 3D US tumor volumes, $r= 0.004$; $p= 0.987$ (Figure 3.20).

3.12 Relationship between OPN and clinical estimate of tumor volume

The relationship between plasma OPN level and the estimate of tumor volume obtained from clinical measure was examined for all patients, at each cycle of neoadjuvant therapy. No statistically significant correlation between clinical estimate of tumor size and OPN level was found at cycles 1-7. Figure 3.21 shows a statistically strong correlation between OPN and clinical estimate of tumor measure at cycle 8 of neoadjuvant treatment for five patients ($r= 0.911$; $p= 0.031$).

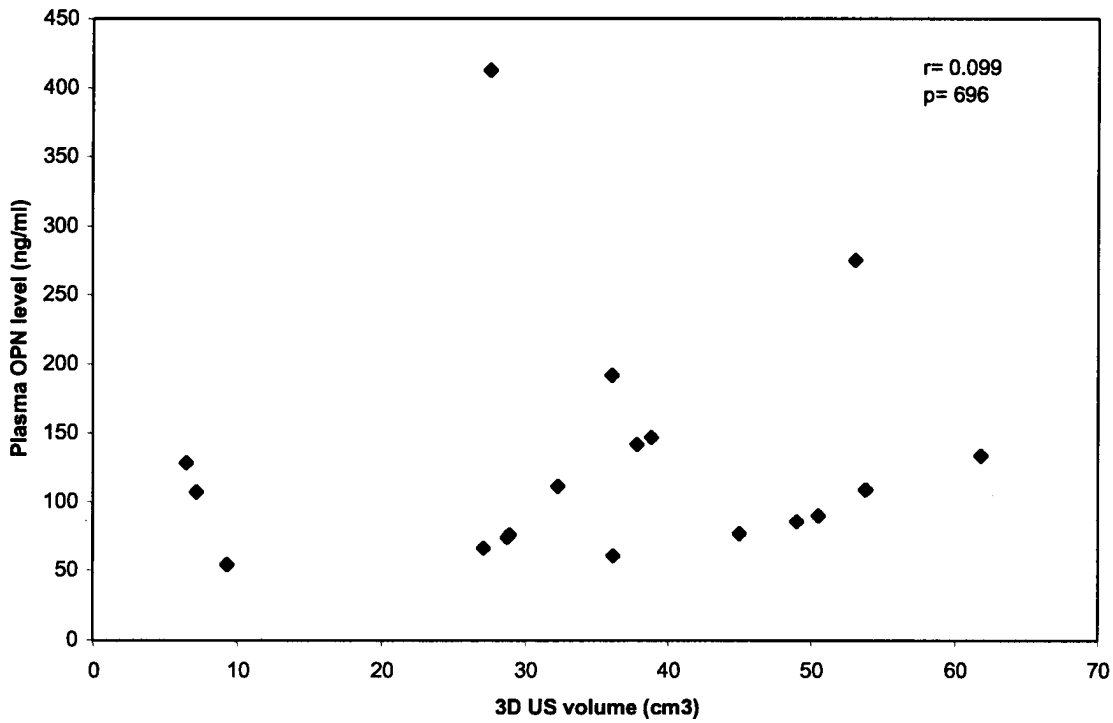

Figure 3.19 Correlation of 3D US measurement of tumor volume with plasma OPN level at cycle 1 of neoadjuvant therapy. No evidence of an association between 3D US and OPN levels at cycle 1 was found (r= 0.099; p= 0.696).

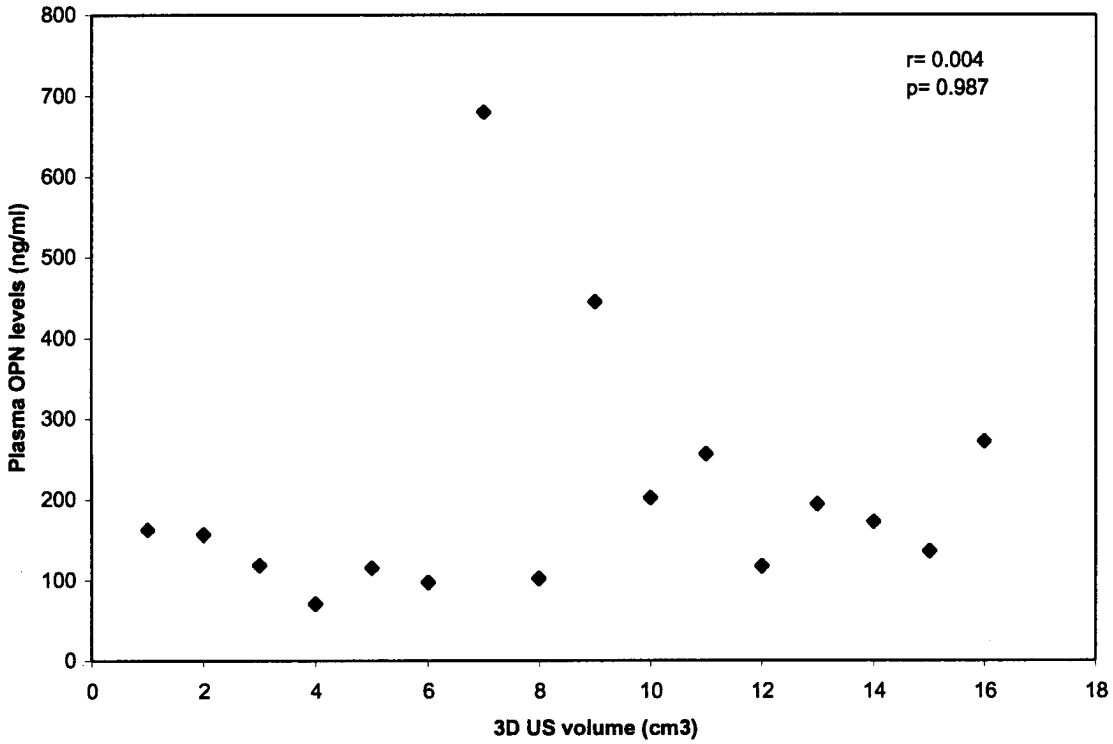

Figure 3.20 Correlation of 3D US measurement of tumor volume with plasma OPN level at cycle 8 for 16 LABC patients. No correlation was found (r= 0.004; p= 0.987).

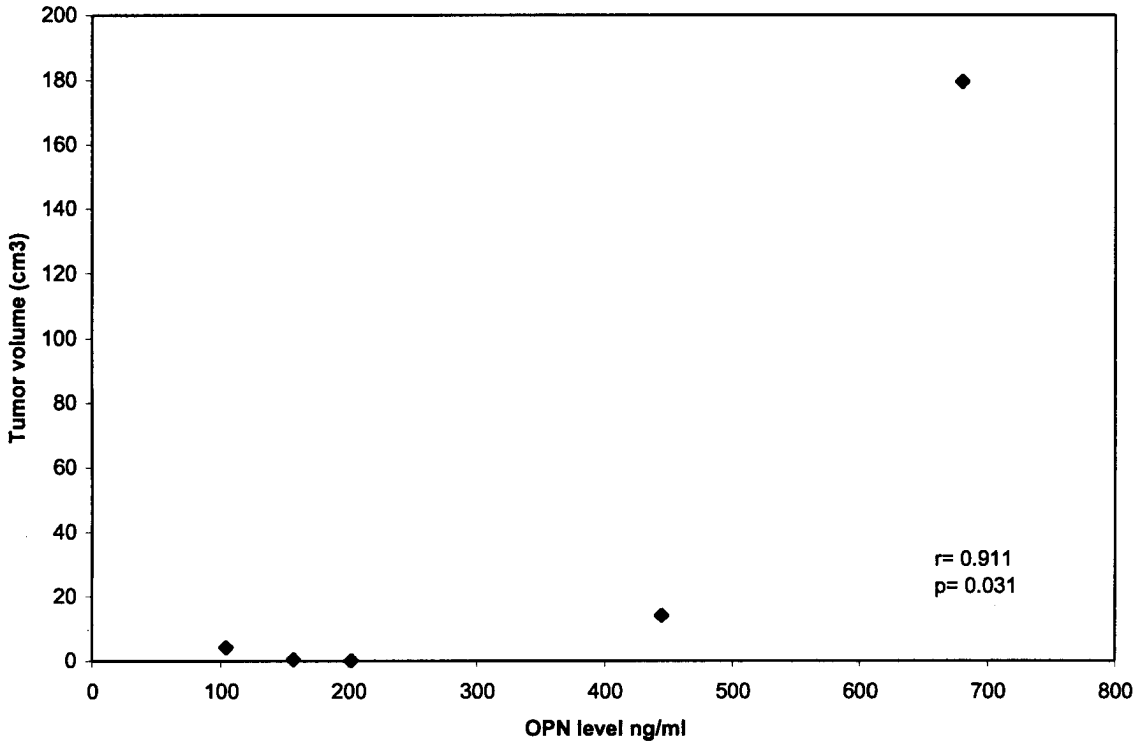

Figure 3.21 Correlation of plasma OPN level with clinical estimate of tumor volume for 5 patients at final cycle (cycle 8) of neoadjuvant treatment. Statistically strong correlation was found (r= 0.911; p= 0.031).

Chapter 4: Discussion

4.1 Thesis Summary

Locally advanced breast cancer (LABC) is the most advanced stage of non-metastatic disease representing 15% of all breast cancer cases. The major clinical challenge associated with these patients is that the majority will have disease relapse and develop distant metastasis, leading to eventual death (Giordano *et al.*, 2003). The form of treatment these patients receive is neoadjuvant therapy. One of the problems with neoadjuvant therapy is the limited ability to clinically detect tumor response to this treatment. Tumor response is an important predictor of prognosis and overall survival for this patient population, so accurate methods are required. By being able to assess tumor response clinically, more accurate and tailored treatment decisions can be made for the individual patient, based on if they are responding or not. Therefore, there is a need to have more precise methods to closely monitor the patient's response to neoadjuvant therapy. In this current study, two novel methods were investigated for monitoring tumor response to neoadjuvant chemotherapy for patients with LABC. These methods were measuring plasma OPN levels and measuring tumor volumes using 3D US imaging over the course of eight cycles of neoadjuvant chemotherapy.

4.2 Discussion of results

4.2.1 Plasma OPN levels throughout neoadjuvant therapy

Of the nineteen patients enrolled in this current study only 10% had a complete response to neoadjuvant treatment (Figure 3.5). The majority or 63% of LABC patients treated in this study with neoadjuvant therapy had a partial response to this form of treatment, with only 5% having no response and 21% having disease progression. These

results are consistent with previous work. It was expected that the majority of patients would have some response to neoadjuvant chemotherapy, as Valero et al. (1996) indicates that 50%-95% of patients with LABC achieve a response when treated up front with neoadjuvant chemotherapy. The literature also indicates that only 3.5%- 30% of patients will have a pathological complete response following neoadjuvant therapy, which is similar to what we found (Taghian *et al.,* 2008). However, our results show that 21% of the LABC patients progressed while receiving neoadjuvant therapy, which is a larger percentage than expected. Studies have previously indicated that fewer than 5% of patients will progress while undergoing neoadjuvant chemotherapy (Taghian *et al.,* 2008). This is a difference that should be investigated because, for the patients who did not respond, their locoregional treatment was delayed. However, these results indicating that the majority of patients did have a partial response, may in fact be due to the small sample size used in the study.

In the treatment of cancer, chemotherapy drugs are used to kill cancerous cells. However, most anti-cancer drugs used are designed to kill growing cells, so they therefore target and destroy actively dividing normal cells in addition to the cancerous ones. The result is that patients can develop a variety of pathologies and side effects altering the functioning of their immune system. OPN, a secreted phosphoprotein, is elevated in various tissues during some stress-responsive physiological situations. Our results show that during neoadjuvant chemotherapy, as the patients receive additional cycles of chemotherapy, there is a statistical difference in the levels of plasma OPN over the course of treatment, with a statistically significant increase in OPN levels at later cycles (7 and 8) compared to earlier cycles (1 through 6). As chemotherapy is

administered, this increase in plasma OPN may be due to the increased OPN expression as a result of the body's stress response, which may in turn involve the inflammation/immune phenomena. In both normal and pathological conditions, OPN is up regulated in the cells of the immune system. This protein is known to be secreted by activated T cells, macrophages, and natural killer cells (Denhardt *et al.,* 2001). Natural killer cells are lymphocytes, which like cytotoxic T cells, attack and kill tumor cells. Macrophages arrive at the tumor site and are responsible for cleaning up and eliminating dead cells and/or cellular debris which has resulted from chemotherapy treatment. During the immune response, immune cells are brought to the site of injury to disinfect, clear debris and stimulate healing and it is believed that OPN plays an important role in this early process (Giachelli *et al.,* 2000). The literature further shows that an increase in OPN expression in these immune cells allows for increased macrophage adhesion, migration, cytokine release and phagocytosis, which are all important events of the immune and inflammatory response (Giachelli *et al.,* 2000). Crawford et al. (1998) demonstrated the importance of OPN expression in immune responsiveness to tumor, by showing that in OPN null mice, the number of macrophages responding to chemical carcinogen-induced squamous cell carcinoma was decreased compared to wild type mice which expressed OPN. Thus, one possible explanation for the increase in plasma OPN levels over the course of neoadjuvant treatment may be the activation of the body's immune responses during chemotherapy.

In addition to activated immune cells expressing elevated levels of OPN, OPN is also detected in primary breast tumors (Brown *et al.,* 1994). Brown et al. (1994) suggested that the OPN detected in a primary tumor may be due to secreted OPN from

host macrophages, which binds to and is taken up by the tumor cells. However, there is additional evidence that mammary carcinoma cells are able to produce their own OPN (Tuck *et al.,* 1998; Wai *et al.,* 2004). OPN is known to act as a chemoattractant for various cell types, including macrophages and T cells, which may then result in these inflammatory cells being attracted to the OPN secreting primary tumor (Furger *et al.,* 2001; Denhardt *et al.,* 2001). If the immune cells are toxic to the tumor, then this can result in the tumor cells' own death. However, other work suggests that tumor-derived OPN may function in a very different manner, leading to increased survival of the primary tumor. The literature suggests that tumor-derived OPN is secreted in order to provide the primary tumor with a growth advantage by helping it evade the immune system (Wai *et al.,* 2004; Crawford *et al.,* 1998). It is suggested that this is accomplished by the tumor derived OPN inhibiting macrophage function and therefore increasing tumor growth, providing a complex, antagonistic role for OPN, highly dependent on the source (cell of origin) and hence form of OPN (Wai *et al.,* 2004; Crawford *et al.,* 1998). Crawford *et al.* (1998) suggest that tumor-derived OPN has the ability to keep macrophages, which have infiltrated the tumor, in an inactive, resting state. Therefore, it may be possible that the increased plasma OPN levels observed in patients over the course of neoadjuvant chemotherapy is from two different sources, OPN expressed by immune cells working with the chemotherapy to target the primary tumor and secretion by the primary tumor itself to help it survive. Further work is required in order to fully understand the significance of the increased plasma OPN seen in patients undergoing chemotherapy, in terms of its effect on immune response, repair processes and effects of the tumor itself and hence response to treatment.

It has been previously reported by our group, that for healthy women, plasma OPN levels are found to range from 22-122 ng/ml and based on this, a level of 123ng/ml has been used as the upper limit of normal (Bramwell *et al.*, 2006). Anything above this value has therefore been described as elevated. Bramwell et al. (2006) published that in a group of metastatic breast cancer patients, 63% of the women had elevated plasma OPN levels at baseline. While plasma OPN levels thus have been reported to be elevated in women with metastatic disease, levels for patients with locally advanced disease (bearing large tumors but not metastatic disease) had not been previously studied. Our results show that in contrast to the metastatic patients' OPN levels, at baseline most LABC patients (61%) have plasma OPN levels that are not elevated above the upper level of normal, 123ng/ml (Figure 3.3). However, over the eight cycles of treatment, there does appear to be a trend towards an increase in the percent of patients with elevated OPN levels at each subsequent cycle. At the final cycle of treatment, the majority of the patients (63%) had elevated OPN levels (Figure 3.4). At some of the early cycles (2, 3 and 5) of treatment, the percent of patients with elevated levels is statistically lower than compared to the late cycles (7 and 8) (Figure 3.4).This work therefore provides new information about plasma OPN levels in women with advanced local disease but without metastatic disease, in that baseline OPN levels are generally not elevated, as they often are in metastatic patients.

Previous studies have looked at the relationship between plasma OPN levels in patients with metastatic breast cancer and their overall survival. Bramwell et al. (2006) reported that baseline OPN levels were inversely and significantly associated with survival. Patients with elevated OPN levels had a poor survival despite treatment for the

disease, when compared to patients with normal OPN levels. This association of increasing OPN levels over time with poor prognosis implicates the use of monitoring OPN levels sequentially, in order to help make treatment decisions and determine response. This current study examined this novel role for plasma OPN in a group of LABC patients. Our results show that 50% of the LABC patients with OPN levels elevated at baseline had no response to treatment or disease progression while receiving neoadjuvant therapy, whereas of the patients that had a complete or partial response to treatment, 36% had elevated baseline OPN levels (Figure 3.6). For patients having a complete or partial response to treatment, the median OPN level at baseline was in the "normal range", 99 ng/ml, whereas for those individuals having no response or progressive disease, the median baseline OPN level was found to be slightly elevated, at 127 ng/ml. These differences, although clear trends, were not statistically different within the power of this study. This trend does suggest that for patients with higher baseline OPN values, there may be an association with a worse response to treatment, although this would require further study with a larger sample size to prove statistically. A clear trend in changes of OPN levels over treatment was observed with a statistical difference in OPN levels at cycle 7 and 8. Although it was observed that the majority of patients did have elevated OPN levels at the end of treatment, of those with the highest levels, there was a trend towards a worse response and therefore, further work must be done to determine if this difference is associated with final treatment response.

In addition, we also looked at the relationship between final OPN levels and final response in Figure 3.8. As noted above, for the last two cycles of neoadjuvant therapy, there was a significant increase in all patients' OPN levels. However, patients who had no

response to treatment or disease progression while on treatment, had a higher median final OPN level (172ng/ml), compared to patients that had a better response to treatment (157 ng/ml). This difference was not statistically different within the power of this study, although it is clinically interesting and worthy of further study. In order to determine if this trend really is of statistical significance, additional patients should be included in the study to further investigate.

4.2.2 3D US volume measurement throughout neoadjuvant therapy

One of the disadvantages of "up front" neoadjuvant therapy is the problem of detecting and monitoring tumor response to treatment. The only standard method of doing this is by a clinical breast exam of the primary tumor at each chemotherapy visit. As the tumor begins to shrink or break up and scar tissue begins to accumulate, this can pose problems and lead to inaccurate measures of the tumor. Overestimates can occur when the extent of the scarred area extends beyond the limits of the regressing tumor. Alternatively, underestimates can occur if the tumor diffusely infiltrates beyond the limits of the palpable mass, without significant desmoplastic stromal response (such that the "true" advancing front of the tumor is perhaps non-palpable). Accurately assessing tumor response is important, because individuals that have a complete response clinically or pathologically have a much better outcome after treatment (Giordano *et al.*, 2003). The main problem with the clinical exam used to estimate tumor volume is that this measure does not correlate well with pathological response after surgery. In addition, another problem that was observed in this study was that the clinical measure was not always obtained, due to the tumor becoming unpalpable, or tumor boarders difficult to find. In

the current study we therefore looked at using 3D US imaging, in order to more accurately monitor tumor volume changes over the course of therapy.

With 3D US, the volume of a structure can be examined in real time and at low cost, right in the clinic. 3D US has been shown to be an effective and accurate method for imaging tumors (Inoue *et al.,* 2005). In addition, the 3D US system that we used for imaging and reconstruction of volume in this current study, has been previously shown to accurately measure volumes of known objects (DeJean *et al.,* unpublished data). As expected, our results show that over treatment, we were able to detect a decrease in median tumor volume for the entire patient population, using the 3D US system (Figure 3.9). In nine of the cases however, tumor volume at the final cycle was overestimated compared to the final pathology. It should be noted that after this final 3D US, one more cycle of chemotherapy was administered followed by surgery, up to one month later, which could have resulted in the overestimate at cycle 8. In addition, as the tumor regresses and scar tissue accumulates, it may impede imaging, due to the presence of a shadow around the remaining tumor volume. In six of the cases, tumors at final cycle were underestimated with the 3D US system, when compared to the final pathology after surgery. In all but one of the underestimates, tumors were found to be large (ranging from 179 cm^3 to over 1000 cm^3) and thus could have prevented the entire structure from being adequately seen on the 3D US screen.

In breast cancer, at time of diagnosis, tumor size is routinely determined by mammography, 2D US or MRI imaging tools. These tools, although not ideal for routine sequential imaging to follow tumor size, allow the medical oncologist to determine the extent of disease at baseline and devise an accurate treatment plan for the patient. In this

study we used these diagnostic measures to determine the volume at time of diagnosis. This was done by using the largest dimension of the tumor (from mammography, 2D US, or MRI) and calculating a volume from it. 3D US imaging has been documented in the literature to be a convenient and accurate method for measuring normal anatomy, as well as visualization and measurement of pathologies (Fenster *et al.,* 2003). With such accuracy, we investigated in this current study if the 3D US system would be useful in predicting patient's response to neoadjuvant treatment. Our results indicate the there was no correlation between diagnostic baseline volume (as determined by mammogram, 2D US or MRI) and 3D US baseline volume, or final volume (from surgical pathology) with final volume from 3D US (Figures 3.13 and 3.17, respectively). In comparison, there was found to be a good statistical correlation between diagnostic tumor volume and baseline clinical measure (Figure 3.12) and a strong statistical correlation between final tumor pathology and final clinical measure (Figure 3.16). It is important to note that at baseline, the medical oncologists have the diagnostic measure of tumor volume when they initially examine the patient and make their clinical estimate of size, so there was no way of controlling for bias. In addition, with respect to clinical estimates, there was a lack of estimates made for all patients at various cycles. Therefore, this would have affected the correlations because there was such a small patient population that could be used. In comparison, all patients received a 3D US at every cycle, so for correlations involving this procedure, all nineteen patient's tumor volumes could be included. For example, for clinical estimates at baseline, only 14 patients could be used for correlation with diagnostic volume, as opposed to all 19 patients for baseline 3D US correlation with diagnostic volume, as 5 patients were not given a clinical exam at initial visit and only

diagnostic volume was used for treatment decisions. In addition, only 7 patients had a final clinical volume estimate documented, which could therefore be used to correlate with final tumor pathology, as opposed to 18 patients that were used for analyzing final 3D US with final pathology. These inconsistencies in the number of patients used for correlating a relationship may have affected the results. An extended study, with larger numbers of patients and a more even distribution between groups would therefore be desirable. It is interesting to note that there was a strong correlation found between final 3D US imaging and final clinical volume, but again only 7 patients were used, due to the lack of clinical estimations (Figure 3.18).

In the case of patients with multicentric tumors, when imaging with 3D US, only the largest tumor structure was imaged. This therefore would have resulted in an underestimate of residual tumor volume and this accordingly would have affected the correlation of final 3D US volume with final volume from pathology. From pathology, to determine the final volume of multicentric tumors, the largest dimension of each individual tumor was used to calculate volume and then all volumes were summed together to get the overall volume. This results in a potential overestimate of the tumor volume and could have affected the correlations as well. Thus, in future studies, a more accurate and concise method of imaging multicentric tumors and reporting their final tumor volume after neoadjuvant therapy is required. This problem of assessing tumor pathology after neoadjuvant therapy is a common issue. After neoadjuvant treatment, surgical specimens from the patients may be difficult to interpret, especially when there has been a good response to treatment (Pinder *et al.*, 2007). Future studies, which include

a larger patient population and ensuring accurate clinical estimations are recorded at every cycle of treatment are suggested, to further investigate this relationship.

4.2.3 Relationship between plasma OPN levels and 3D US imaging

The goal of the current study was to determine if measurement of plasma OPN levels and measurement of tumor volumes using 3D US imaging could more accurately predict the response LABC patients have to neoadjuvant therapy. In order to determine if there was any relationship between these two modalities, OPN levels and 3D US tumor volumes at all eight cycles of treatment were analyzed. Our results indicate that there is very little evidence of a correlation over the 8 cycles of neoadjuvant therapy (Figure 3.19). Throughout treatment we have seen that plasma OPN levels increased over time, whereas with chemotherapy treatment, the majority of patients did have a response, such that the tumor decreased in size.

We also looked at the relationship between clinical measure of tumor volume and OPN levels. Again, no correlation between the two modalities was found for cycles 1 through 7. However, at cycle 8, there was found to be a strong statistically significant correlation (Figure 3.21). This result must be interpreted with caution, as only 5 patients were used for correlation.

4.3 Conclusions

1. This study provides novel evidence that at baseline, 39% of LABC patients with a primary tumor have elevated plasma OPN levels, in comparison to 63% of newly diagnosed metastatic breast cancer patients (Bramwell *et al.,* 2006). This study also shows that for the LABC patients, there is an increase in the percent of patients with elevated OPN levels at cycle 8, with 63% having elevated levels at

the end of treatment. There is also evidence that between the early and late cycles of treatment, there is a statistical difference in the percent of patients with elevated levels.

2. This study provides the first evidence that for LABC, patients' plasma OPN levels increase over the course of treatment, with a significant statistical increase at the final cycles of treatment.
3. A partial response to neoadjuvant treatment was reached by the majority of patients after treatment, with progressive disease being the next most common outcome.
4. For patients with elevated baseline OPN levels, there was a trend towards an unfavorable response to treatment, with these individuals having either no response or progressive disease.
5. Although the majority of patients did have elevated levels of OPN at the final cycle of treatment, there was a trend indicating that patients having higher OPN levels at this point had a worse response to treatment.
6. 3D US measure of tumor volume resulted in overestimates and underestimates of tumor volume, when compared to final pathology. Also, similar over- and underestimates were seen with clinical measurements when compared to final pathology. Evidence indicates that 3D US imaging detects a trend of decreasing tumor volume over the eight courses of neoadjuvant therapy.
7. A trend was found that may indicate that patients who have a smaller tumor volume detected by 3D US imaging at baseline, may be more likely to have a complete or partial response to neoadjuvant therapy.

8. Evidence showed a statistical correlation between the baseline clinical estimate of tumor volume with diagnostic volume, as well as a correlation between final clinical estimate and final pathology after treatment. No correlation was found between 3D US baseline and final tumor volume with diagnostic and final tumor volume from pathology.
9. Evidence of a strong statistical correlation between clinical estimates of final tumor volume with tumor volume from final pathology was found. Evidence shows that with the use of 3D US for measuring tumor volume, there was no statistical correlation with tumor volume from final pathology, even though there was a strong correlation between final 3D US tumor volume and final clinical tumor volume.
10. No evidence of a correlation between the levels of plasma OPN and tumor volumes measured by 3D US imaging over the eight cycles of chemotherapy was found.
11. A strong statistical correlation was observed between OPN levels and clinical tumor volumes at cycle 8. Other cycles showed little evidence of a correlation.

4.4 Future research

This current research work has provided novel data that has not been examined previously, with regard to the levels of plasma OPN in breast cancer patients being treated with neoadjuvant therapy. In addition, a new method of imaging was investigated, to see if sequential changes in tumor volume could be detected and used to assess response to therapy. This pilot study shows possible promise with respect to the trends observed with the measurement of plasma OPN levels. For example, trends for baseline

OPN vs. final response and final OPN levels vs. final response indicate that patients with lower OPN levels (at both time points) seemed to have a better response to treatment, although this was not statistically significant, it appears to be clinically important. In order to determine if these trends are significant, more individuals need to be added to the study to increase the patient population and hence the power of the study. If these trends are found to be significant, future work could be used to help clinicians determine response to treatment prior to its termination. This would be important in helping to make treatment decisions and would possibly help to prevent delays in locoregional therapy and/or surgery, for non-responding patients.

With the comparison of 3D US tumor volumes with final response, no differences were seen between baseline or final volumes and the overall response. However, a trend was apparent in that a smaller 3D US volume at baseline and at final cycle resulted in a better response.

Future work for this study should also examine long term follow-up of the patients. Disease-free and overall survival from surgery to time of relapse or death should be considered. It would be useful to know how OPN levels behaved once the primary tumor was removed from the body and if levels returned to healthy ranges for all patients no matter what their response to treatment was. Furthermore, it would be interesting to determine the relationship of the response to neoadjuvant therapy to overall and disease free survival, as well as if baseline and/or final OPN levels are associated with survival.

As plasma OPN levels were found to increase over the course of neoadjuvant therapy, the source of this OPN would be of interest. Is this increased plasma OPN the result of increased tumor burden, or the body's response to the stress of disease and

chemotherapy? To determine if the primary tumor is itself increasing the levels of OPN due to treatment, *in vitro* work could be done. Use of cells that express elevated OPN compared to those that do not, for treatment with the same (neoadjuvant) chemotherapeutic agents *in vitro*, one could determine if the cells showed altered expression of OPN, and if this was associated with altered invasiveness.

Finally, with respect to 3D US, although the literature indicates that it is an ideal imaging method, our results show that it did not correlate with the final tumor size from pathology. Difficulties with imaging tumors due to scar tissue, extremely large tumors, and multicentric tumors could have led to some of the inaccuracies in measurements reported here. 3D US in this study was shown to be easily and conveniently brought into the clinic, allowing the opportunity for real time images and measures which has the ability to assist the physician. This non-invasive, inexpensive imaging modality therefore has the ability to provide many benefits. Future work focusing on how to best and most efficiently deal with the problems encountered in this current study need to be addressed before 3D US can be routinely used in the clinic.

In conclusion, this pilot study has shown promise with respect to using OPN levels as a marker for monitoring response to neoadjuvant chemotherapy for patients with LABC. Our work has shown the feasibility of such a study, as we were able to enroll nineteen patients and routinely follow each one in the clinic over their eight weeks of chemotherapy. This indicates that future work, with expanded numbers, are warranted, especially for further investigation of OPN levels, so that the clinical management of these LABC patients may be potentially improved.

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