PREDICTING LOCAL RECURRENCE FOLLOWING BREAST CONSERVING THERAPY FOR EARLY STAGE BREAST CANCER: THE SIGNIFICANCE OF A NARROW (LESS THAN OR EQUAL TO 2MM) SURGICAL RESECTION MARGIN

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ABSTRACT

Introduction

Controversy continues over the extent of surgical resection margin required to minimize the risk of local recurrence (LR) in breast conserving therapy (BCT) for stage I and II breast cancer. This thesis explores whether or not a narrow (less than or equal to 2 mm) but negative resection margin in BCT for stage I and II breast cancer affects LR.

Methodology

To address the question, all patients registered at the Saskatoon Cancer Center between January 1, 1991 and December 31, 2000 with a diagnosis of stage I or II invasive duct carcinoma of the breast treated with BCT were examined. All charts and pathology reports were reviewed with a review of the pathology for all cases where the resection margin was unclear in the original report. Other factors know or thought to effect LR (age, radiation boost, grade, extensive duct carcinoma in situ, ER/PR receptor status, tumor size, and systemic adjuvant therapy) were considered in the statistical analysis.

Results

Amongst the 200 narrow margin cases 19 LR were detected (19/200=9.5%) while 52 LR were detected in the 491 wide margin cases (52/491=10.6%). This difference was not statistically significant.

Conclusions

A narrow (less than or equal to 2 mm) surgical resection margin does not result in an increase in local recurrence compared to a surgical resection margin greater than 2 mm in breast conserving therapy for early stage duct carcinoma and does not warrant re-excision.

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Dedication

This thesis is dedicated to the many individuals who have struggled with a cancer diagnosis, their families and friends, as well as those who work tirelessly to help them both on the front lines and in the realm of research.

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CHAPTER 1
INTRODUCTION

1.1 Overview

The research question that is being addressed in this manuscript arose from the author's clinical surgical practice. For many years the standard of care for performing breast conserving surgery in Saskatoon was to remove the cancer with at least some surrounding tissue. Subsequent radiation treatment was given to minimize local recurrence. During that era the patient was submitted to a re-excision only in the event of a positive surgical resection margin. More recently newer radiation oncologists at the Saskatoon Cancer Agency began to request a re-excision for all tumors in which the original margin was less than 2 mm. This change in practice resulted in many more women undergoing a second or even third operation. The evidence to support that practice change was insufficient to warrant the change from the author's perspective and served as the impetus for conducting this research.

Current evidence suggests that breast conserving therapy (BCT), which includes wide local excision and post-operative adjuvant irradiation, is equivalent to mastectomy with respect to overall survival for early stage breast cancer. There is, however, an increased local recurrence rate with BCT in the order of approximately 1% per year compared with an overall local recurrence at 10 years with modified radical mastectomy of about 1%. Subsequent management of a local recurrence frequently results in a completion mastectomy with an attendant decreased quality of life. There is also evidence to suggest that while the overall survival between patients who undergo a mastectomy or BCT is equivalent the survival of the subset of individuals who do have a local recurrence is lower. Minimizing the incidence of local recurrence would therefore be desirable.

The conventional definition of a negative surgical margin in North America, as defined in the original work done by Fisher, is “no tumor at the inked margin”. European trials conducted around the same time required a wider surgical resection margin. The Milan trial required a 2-3 cm margin while the European Organization for Research and Treatment of Cancer (EORTC) trial required a 1 cm surgical resection margin. In recent years there has been some concern expressed that a narrow surgical resection margin (defined as less than or equal to 2mm) may be associated with an increased local recurrence rate compared with a surgical resection margin greater than 2mm. The literature in this area is inconclusive. This thesis work is designed to contribute evidence towards the resolution of this issue.

The article that resulted from this thesis work and was published in the Journal of Surgical Oncology in March of 2011 is found in Appendix A.

1.2 Objective

The objective of this thesis is to determine if a narrow (less than but equal to 2 mm), but negative, surgical resection margin in BCT done for early stage breast cancer results in an increase in local recurrence.

1.3 Hypothesis

The hypothesis is that a narrow (less than or equal to 2mm) surgical resection margin in BCT done for early stage breast cancer does not result in an increased local recurrence rate.

CHAPTER 2
LITERATURE SURVEY

2.1 Introduction

Two separate critical reviews were conducted to support this thesis work. Theories about what causes cancer are critically necessary to inform research questions that have to do with the management of cancer and as such the first review was, necessarily, a review of the current theories of oncogenesis. For this survey the Pub Med and Google Scholar databases were searched using the key words oncogenesis, tumerogenesis, and carcinogenesis and limited to the reviews in the last 5 years in the English language. A total of 4,329 citations were generated, of which 237 were used in the critical review of the theories of oncogenesis.

The second review was a more specific review of the evidence that currently exists about local recurrence in early stage breast cancer patients treated with breast conserving therapy. This review was conducted to inform the specific research question that makes up the main portion of this thesis work. For the survey about local recurrence in early stage breast cancer the Pub Med database was first searched using the following key words: breast neoplasms, neoplasm recurrence, local, and mastectomy, segmental. This search strategy generated 6768 citations of which 983 were considered relevant to the subject matter and reviewed in detail.

2.2 Literature Survey of Oncogenesis

Oncogenesis refers to our understanding of what causes cancer. Sonnenschein and Soto lament the vast quantities of research performed in the quest to eradicate cancer done in the absence of a theoretical framework. (1) They quote Leslie Foulds as commenting that "experimental analysis has produced an alarming mass of empirical facts without providing an adequate language for their communication or effective concepts for their synthesis" in making their case that the premises and perspectives that researchers bring to their research should be

made *a priori* "as premises are not testable, and data are not free of the theoretical frame used to gather them".

By far the most well know and accepted theory of oncogenesis is the gene mutation theory. Peyton Rous laid the groundwork for this theory in 1911 when he identified a spindle cell sarcoma in chickens that was transplantable from one chicken to another.(2) A filtrate of a malignant tumor was used to transmit the cancer from one bird to another. In time it was discovered that the rous sarcoma virus (RSV) was the infectious agent responsible. Years later Bishop hypothesized that "normal cells may bear the seeds of their own destruction in the form of cancer genes. The activities of these genes may represent the final common pathway by which many carcinogens act. Cancer genes may not be unwanted guests but essential constituents of the cell's genetic apparatus, betraying the cell only when their structure or control is distributed by carcinogens."(3) He hypothesized that cancer-causing genes (oncogenes) that were carried by tumor causing viruses had counterparts in the genomes of all vertebrate cells. He termed these normal counterparts proto-oncogenes. A multitude of studies have since demonstrated the existence of a family of viral oncogenes that can be transmitted either via DNA or RNA viruses.(4) DNA viruses replicate their DNA along with host genome and promote neoplastic transformation in conjunction with environmental and genetic factors. RNA tumor viruses on the other hand integrate their RNA genomes into the genome of the host cell inducing neoplastic transformation by integrating its genome near the coding sequence for a proto-oncogene. Through multiple rounds of infection and genome replication mutations occur in the proto-oncogene resulting in neoplastic transformation. Bishop's initial theory has since been refined but the basic tenant is that carcinogens of one sort or another interact with DNA resulting in irreversible changes in the gene (point mutations), which predispose the cells to malignant

transformation. The gene mutations occur in 2 classes of regulatory genes, oncogenes (positive regulators) and tumor suppressor genes (negative regulators). Modifications of the gene mutation theory include Knudson's two hit hypothesis, and several multistep hypothesis such as those proposed by Weinberg, Barrett and Vogelstein.(5-8) Of these the Vogelstein model has gained the most popularity. In it Vogelstein and colleagues postulated a progressive model, which includes both activation of oncogenes and the loss of tumor suppressor genes. Feinberg introduced the concept of epigenetic changes that allows for non-mutational stable changes to occur in the cellular genome and that can contribute to cancer transformation.(9)

Duesberg et al. advanced a new theory that suggests that a carcinogen initiates carcinogenesis via a preneoplastic aneuploidy event.(10) According to this theory, while the majority of aneuploidy cells die following cell division the rare one survives. The surviving aneuploidy cell then initiates an "autocatalytic karyotype evolution" that generates new chromosomal variants including, eventually, a neoplastic aneuploidy cell.

Early work done by McCullock and Till resulted in the development of what McCulloch terms "modern hematology", which includes the concept of the stem cell.(11) In 1963 they suggested that stem cells have two defining properties—self renewal and differentiation.(12) Initially it was thought that stem cells functioned only in obligatory renewal systems such as the hematopoietic, epithelial mucosal or skin systems. More recently findings from several laboratories demonstrate the existence of adult stem cells with the potential to differentiate and produce the functional cells of several organs.(11) These cells are potential targets for carcinogenic events.

In 1997 Bonnet and Dick first coined the term cancer stem cells (CSC) to describe a small population of leukemic cells that have the ability to self-renew.(13) With further evidence to

support the concept a new theory of oncogenesis—the cancer stem cell theory—has emerged. The theory states that only a small subset of cancer stem cells exist within a tumor and cause the propagation of a given tumor.(14) These cancer stem cells have the capacity to self-renew and to form the heterogeneous lineages of cancer cells that comprise the tumor. In the CSC theory it is believed that non-tumorigenic cells in a cancer are derived from parent tumorigenic cells in a hierarchical and stable manner that parallels in concept the development of differentiated cells from stem cells in normal tissue development.(13) The CSC theory does not, as the name implies, refer to the cell from which the cancer arises. In fact the CSC theory does not require that tumorigenic cells be similar phenotypically, genetically, epigenetically or functionally to normal stem cells of the same organ. What it does require is for tumorigenic cells to be infrequent, to be capable of generating both more tumorigenic cells as well as the larger population of non-tumorigenic cells in a stable and hierarchical manner and to be separable from non-tumorigenic cells. In the CSC theory it is thought that malignant transformation is likely to occur through dysregulation of the self-renewal pathways.(15)

There is significant interest in, and evidence to support, the CSC theory. Several implications flow from this theory. CSC share many properties with normal stem cells, namely a self-renewal capacity, resistance to radiation and many chemotherapeutic agents and potentially the ability to interconvert (the ability to change between different functional states—changing their proliferative and differentiation fates in response to environmental causes).(16) Self-renewal is the property that allows stem cells to produce at least one progenic cell with similar developmental potential. When stem cells divide one daughter is an exact copy of the original and the other differentiates. It is interesting that many of the genes responsible for self-renewal are also oncogenes and many genes that inhibit it are tumor suppressor genes.(13) Stem cell

research has shown us that self-renewal is controlled by several processes such as the hedgehog pathway, the notch pathway, Wnt signaling and NF-kB signaling.(13-15)

Sonnenschein and Soto suggest that all of the above theories of oncogenesis are variations on a theme, that theme being cell proliferation control as the cause of cancer. (1) They then suggest that there exists a new emerging theory centered at the tissue level and called the tissue organizational field theory (TOFT) which views oncogenesis more like "a process akin to organogenesis gone awry." The premises underlying this theory are that oncogenesis is a problem of tissue organization, comparable to organogenesis during early development, and that proliferation is the default state of all cells. In the TOFT model carcinogens disrupt reciprocal interactions between cells that maintain tissue organization, tissue repair and local homeostasis and result in cells being allowed to exercise their innate ability to proliferate and migrate.

The various theories of oncogenesis need not be thought of as mutually exclusive. As Sonnenschein and Soto point out there is a significant amount of overlap in the various somatic mutation theories.(1) Even between those various theories and TOFT there is considerable overlap emerging when one considers the epigenetic contributions to oncogenesis in the gene mutation theory and the microenvironment considerations in the CSC theory. All of these theories agree that the 5% of malignancies that are hereditary cancers are caused by germline DNA mutations. It is quite conceivable that, while there appears to be considerable philosophical distance between the different theories, some form of hybrid theory incorporating these will emerge. The very heterogeneous and complex nature of cancer would make that thinking very congruent. That there is convincing evidence to support each theoretical model also supports that possibility. At the very least information gathered under the rubric of one theory or the other might be used to help inform the overall understanding of the problem.

2.3 Literature Survey of the Evidence Surrounding Local Recurrence in Early Stage Breast Cancer Treated with Breast Conserving Therapy.

2.3.1 History

Halsted proposed that a radical resection was necessary to cure breast cancer. The theory underpinning Halsted's radical mastectomy suggested that breast cancer spreads via lymphatics in an orderly and defined manner and that hematologic spread was of little to no significance.(17, 18) With that theoretical belief Halsted advocated radical surgery, which included removal of the breast, the underlying pectoralis major muscle and the axillary lymphatics. Research, beginning in 1959, led to the formation of an alternative hypothesis of spread that argued that cancer is a systemic disease that involves a complex spectrum of host-tumor interrelations and that variations in local regional therapy are unlikely to substantially affect survival. The differences between the two theories as articulated by Fisher are summarized in table 2.1.(17) In 1971 the National Surgical Adjuvant Breast and Bowel Project (NSABP) launched its B-04 clinical trial. In this trial, first published in 1977, 1700 women were randomly assigned to one of three surgical treatment arms—total (simple) mastectomy and local-regional radiation, total mastectomy alone, and radical mastectomy.(19) Fifteen year follow-up showed no significant difference in treatment failure, distant metastasis or survival. Consequently a 1979 National Institute of Health consensus conference concluded that total mastectomy and axillary dissection should replace the Halsted radical mastectomy as the standard of care.(20) With an understanding that this recommendation reflected a new theory of breast cancer biology the consensus conference went on to recommend that the evaluation of procedures aimed at preserving the breast should be vigorously pursued.

Table 2.1 Differences between the Halsted Theory and Fisher's Alternative Hypothesis

Halsted's Theory	**Fishers Alternative Hypothesis**
Tumors spread in an orderly fashion based on mechanical considerations	There is no orderly pattern of tumor spread
Tumors spread to lymph nodes via direct extension and therefore an "en block" resection is required	Tumor cells travel to lymphatics via embolization
A positive lymph node indicates tumor spread and equates to systemic disease	A positive lymph node is an indicator of a host-tumor relationship that permits the development of metastasis rather than the instigator
Regional lymph nodes (RLN) are a barrier to the passage of tumor cells	RLN do not serve as a barrier to spread
RLN are anatomically important	RLN are biologically important
Tumor does not spread hematologically	Hematologic spread is an important way that breast cancer disseminates
Cancer is autonomous of the patient	Complex host-tumor inter-relationships affect every aspect of the disease
Operable breast cancer is a local regional disease	Even operable breast cancer is a systemic disease
The extent of surgery is the most important factor determining patient outcome	Variations in local regional treatment are unlikely to substantially affect survival
No consideration was given to multicentricity	Multicentric foci of occult tumor are not necessarily a precursor of clinically overt cancer

Fisher's seminal publication of the NSABP-06 clinical trial in the New England Journal of Medicine in 1985 was the first of 10 long term randomized controlled trials, demonstrating that breast conserving surgery (BCS) has equal survival compared with a modified radical mastectomy. (19, 21-30) In his study patients with T1or T2, N0 or N1, M0 breast cancers 4 cm. or less were randomized into one of three treatment arms: total mastectomy, lumpectomy or

lumpectomy followed by breast irradiation. In the NSABP-06 trials lumpectomy was defined as removal of "only sufficient tissue to ensure that the margins of resection were free of tumor."(19)

While it is now generally accepted that breast conserving surgery is equivalent to modified radical mastectomy in terms of survival it was clear from Fisher's first publication that it comes at the cost of increased local regional recurrence.(18, 26, 31-35) In his original report a Cox regression model indicated only three covariates that predicted tumor recurrence—treatment with radiation post operatively, tumors with poor histology and tumors greater than 2 cm. in size.

Because of the way that various authors have dealt with the concept of local recurrence it is difficult to compare the incidence of local recurrence from one publication to another. Some authors include nodal recurrence in their definition while the majority restricts their definition to disease in the breast alone. Still others make a distinction between true recurrences and new primary disease in the breast. This will be discussed in more detail later in this literature review. In the NSABP-06 trial they only considered a recurrence to be a local recurrence if it was the first presentation of recurrent disease. In other words if a given patient developed metastatic disease first and then developed a recurrence in the breast it was not considered a local recurrence. Depending on the publication local recurrence is considered at any of a number of time periods with five, ten, fifteen and twenty year follow-up being common end points. Clearly the length of follow-up will influence the rate of local recurrence but this is often forgotten in the subsequent discussion. With that as background the published local recurrence rates for BCT range from a low of 3-14.3% at five years.(21, 36-44)

A substantial body of literature followed Fisher's 1985 study looking to understand what, if any, factors might predict for local recurrence with a view to either modify the variable itself in such a way as to improve the rate of local recurrence or to modify the selection of patients who

are candidates for BCS. The initial publications consisted of small numbers of cases with short follow-up and weak statistical analysis. With time, studies examining larger data sets with longer follow-up and using multiple variable regression techniques emerged. Initially the variables examined were limited to the concepts already in the literature. These variables were age, tumor grade, tumor size, histological type, the presence of extensive duct carcinoma in situ (DCIS) lymph node status, the use of radiation boost to the tumor bed, lymphovascular invasion, estrogen receptor status, and the presence or absence of multifocality. The possibility of a narrow but negative surgical resection margin being a variable that might negatively affect local recurrence had not yet surfaced in the collective thought of the research community, hence it was not a variable that was examined early on.

In the mid to late 1980's several articles were published that examined surgical specimens that were re-excised after an initial lumpectomy looking for the incidence of residual carcinoma in the re-excised specimen. Overall these studies showed that there was a higher rate of residual carcinoma in re-excised positive or narrow margin lumpectomies compared with lumpectomies in which the initial surgical resection margin was greater than 2 mm.

Subsequently a number of studies including margin status as one of the variables to be examined in the multiple variable analysis were conducted. Originally these studies only considered margin positivity. Starting in 1987 until as late as 2009 articles including positive margin status were published. By early 2000 19 articles had been published showing a strong association between a positive surgical resection margin and local recurrence. Even though a further 9 articles confirming this association have been published since 2000 the consensus opinion by then was that there was adequate evidence to support re-excision of lumpectomies in which the surgical resection margin was positive.

The first article that considered the possibility of a narrow surgical resection margin being associated with local recurrence was an article by R.D. Pezner et al. entitled “to boost or not to boost: decreasing radiation therapy in conservative breast cancer treatment when ‘inked’ tumor resection margins are pathologically free of cancer.”(45) Since then twenty-one other publications have specifically considered narrow or close resection margins and their effect on local recurrence in the ipsilateral breast following BCT. These publications will be examined in some detail subsequently.

Like the issue of narrow margins the potential role that systemic adjuvant therapy might play on local recurrence in BCT was not initially examined. With time more and more early stage women began to receive systemic adjuvant therapy (either chemotherapy or hormonal therapy) and it was only then that the potential role in decreasing local recurrence became evident. The first publication considering this was an article by Fisher in 1989.

Most recently the molecular work with DNA microarray analysis has begun to make its way into the literature as a potential predictor of local recurrence and this will also be examined in some detail.

2.3.2 Definition of Local Recurrence

Unfortunately there is no consistent definition for local recurrence used in the literature, making comparison of studies somewhat challenging. Depending on the publication, local recurrence has included nodal recurrence, skin recurrence, all recurrences in the ipsilateral breast or selective ipsilateral breast recurrences.

Given that the difference between mastectomy and breast conserving surgery is the extent of surgery in the breast, most authors have not included axillary nodal recurrence in their definition of local recurrence. There is no evidence that could be found to support the concept that nodal

recurrence is increased in breast conserving surgery supporting the majority of authors who have not included it in their definition.

A recurrence of malignancy in the ipsilateral breast could, in theory, be due to recurrence of residual disease or a new primary malignancy, a concept first articulated by Veronesi.(46) The literature addressing this question suggests that there is likely a difference in the biological significance of these two theoretical events and an attempt to distinguish between them would be reasonable for that reason alone.(46-49) According to the hypothesis advance by Veronesi, true recurrences are cases consistent with regrowth of malignant cells not removed by surgery and not killed by adjuvant radiotherapy. New primary tumors however are new malignancies arising from residual breast epithelium. Subsequent literature suggests that this concept is likely true with new primary malignancies having a better prognosis than true recurrences. While both types of recurrence are a consideration with BCT only true recurrences would be affected by the extent of resection of the primary tumor assuming that the incidence of new primary tumors is the same whether the person had a small amount of breast tissue removed or a larger one. Komoike et al. used location of the primary and secondary tumor, initial surgical resection margin and other pathology to distinguish true recurrences from new primaries while others used location histology and DNA flow cytometry or just location and histology.(50) Abd-Alla et al. provides us with a useful clinical definition in which an ipsilateral breast recurrence was considered a true recurrence if it was located within 3 cm of the primary tumor bed and had a histological subtype consistent with the primary tumor.(47)

While the current methods described to differentiate a true recurrence from a new primary tumor are useful when the local recurrence is in a separate quadrant of the breast or of a different histology tumor heterogeneity makes it extremely difficult to distinguish the two when the local

recurrence occurs in the same general location in the breast as the primary malignancy and is of the same histology. It is likely that genetic markers will aid in distinguishing true recurrence from new primaries in the future.

2.3.3 Definition of a Narrow Surgical Resection Margin

There is a lack of consistency in the literature around what is called a narrow surgical resection margin adding a degree of challenge to the comparison of publications. While the most common definition is less than or equal to two millimeters some authors have used a one millimeter margin and others have used three millimeters or even more.

2.3.4. Re-excision Studies

The initial re-excision studies conducted by Solin et al., Frazier et al., and Schnitt et al. will be examined closely as they seem to have been significant in informing the narrow or close margin debate early on.

In all of these studies the authors reported the rates of residual carcinoma in re-excision or mastectomy specimens. In Solin's series they examined 185 patients who underwent an excisional biopsy. They found that 51% of the re-excised specimens had residual carcinoma and that a positive initial resection margin carried with it the highest likelihood of residual disease (60%). They concluded however "when inked margins were negative re-excision is not recommended" as the residual carcinoma rate in that group was not high.(51) In Frazier's study they examined both re-excision specimens and mastectomy specimens. If the initial biopsy was positive the likelihood of residual carcinoma was 21/40 (52.5%), if it was close it was 9/28(32.1%) and if it was negative it was 5/19 (26.3%).(52) The Schnitt study of 71 re-excised specimens indicated a higher residual carcinoma rate if there was an extensive amount of DCIS in the initial lumpectomy.(53)

The issue of residual tumor in re-excision specimens continues to garner interest. In Skripenova's 2010 publication he reviewed lumpectomy specimens that had a subsequent re-excision and found a higher rate of residual carcinoma in specimens that had an initial positive margin (44%), versus a less than 1 mm. margin (25%) versus a margin of 1-2 mm (28%) versus a margin greater than 2 mm (16%).(54) In 2006 a similar study conducted by Scopa et al. reviewed 201 lumpectomies that were re-excised and found a 63% residual carcinoma rate in those whose initial margin was positive, a 30% residual carcinoma rate if the original resection was 0-1mm, and a 21% residual rate if the initial margin was greater than 1mm.(55) Sabel et al., Cellini et al., Gwin et al., and Swanson all found no significant difference between positive and narrow margin re-excisions.(56-59)

In all of these studies the number of narrow margin specimens was limited and in all of the studies the cohort was restricted to specimens in which a re-excision was conducted for clinical reasons. As such we do not know what the rate of residual carcinoma would have been in re-excised wider margin lumpectomies and a real comparison cannot be made. In 2007 Kotwall et al. conducted a study in which all patients had either a second excision or mastectomy following their initial lumpectomy.(60) In his study if the initial lumpectomy showed multiple focally positive margins the subsequent re-excision showed residual carcinoma 30% of the time, if it was focally positive 22% of the time, if it was less than 1mm the residual rate was 8%, 15% if the margin was 1-2mm and 4% if the initial margin was greater than 2 mm. Unfortunately, there is no indication in his article whether or not the initial lumpectomy was designed to be definitive or not. Given that all initial lumpectomies went on to further surgery it is likely that the initial lumpectomy was designed only to be diagnostic in which case one would expect there to be a high frequency of residual carcinoma in lumpectomies with positive margins.

These re-excision studies led some to conclude that the need for re-excision of tumors with an initial narrow margin is self-evident. Presumably this explains why a recent survey of oncologists showed that a significant number of them advocated either a 1 or 2 mm surgical margin and some as much as a 3 mm margin or greater.(61) That conclusion fails to take into account the known effect of adjuvant whole breast radiotherapy. It is accepted that, in the absence of radiotherapy, there is a significant (30%) local recurrence rate. Postoperative whole breast adjuvant radiotherapy reduces that risk to the now accepted range of approximately 1% per year. The addition of a boost of radiation to the tumor bed further reduces the local recurrence risk to about 6% over 10 years.

More significantly, in BCT the appropriate question to be asked is not how much residual disease is present after a lumpectomy but rather what amount of lumpectomy is necessary for the residual breast tumor burden to be lowered enough that it is likely to be controlled by adjuvant radiotherapy. The question of the amount of residual disease would become pertinent if consideration was made to forego radiation therapy in selected cases as is currently being discussed by some authors.

2.3.4 Variables Associated with Local Recurrence

2.3.4.1 Radiation as a variable

2.3.4.1.1 Overview

There is little doubt that post-operative adjuvant radiation decreases local recurrence following breast conserving surgery. Breast Conserving Therapy (BCT) in fact refers to a lumpectomy with negative surgical resection margins and post-operative whole breast radiation therapy. This is currently one of two accepted methods of treating early stage breast cancer, the other being a simple mastectomy. In both scenarios some sort of lymph node sampling is still

considered important for prognostication and decision making regarding systemic adjuvant therapy but not for local control.

Beginning with Fisher's NSABP-06 trial that showed a statistically significant difference in local relapse between patients who had a lumpectomy only versus a lumpectomy and post-operative adjuvant irradiation (40% vs. 10% at 8 years) multiple studies have demonstrated the significant benefit of post-operative adjuvant radiation therapy.(62-69) Unlike most variables associated with BCS the beneficial effects of adjuvant radiation has been studied in six randomized prospective trials and subjected to two meta-analysis.(21, 37, 38, 40-42, 70, 71)

2.3.4.1.2 Radiation Dose

The "standard" radiation dose ranges from 45-50.4 Gray (Gy) given to the whole breast. A number of centers advocate for a "boost" of radiation to the tumor bed anywhere from 16-25 Gy. A boost of radiation has been shown to further reduce local recurrence but at a cost of increased fibrosis and a poorer cosmetic outcome. The randomized boost vs. no boost EORTC 22881-10882 trial reported by Bartelink et al. reports a reduction in local recurrence at 10 years from 10.2% to 6.2% in those patients who had a 16 Gy boost of radiation with an associated increase in severe fibrosis of 4.4% vs. 1.6% in the no boost arm.(72) These numbers were statistically significant.

2.3.4.1.3 Radiation timing

A number of articles have been published exploring the importance of the timing of post-op radiation in local recurrence.

In 2002 Hebert-Croteau et al. reviewed the literature and found conflicting evidence. There was only one experimental study, which suggested that a delay to radiation might compromise

local control consistent with a few retrospective studies.(73) Other observational studies suggest otherwise.

Subsequently Ampil noted a trend toward more local and systemic failure when breast irradiation was initiated more than seven weeks following BCS.(74) Fortin et al., Bese et al. and Vujovic et al. also found that delays in post-operative radiotherapy might increase the risk of local recurrence.(75-78) Punglia et al. found a continuous relationship between the interval from breast conserving surgery to post-operative radiation and local recurrence in older women.(79) Benchalal et al., Donato et al and Jobsen et al. all found that it was safe to delay radiation until after systemic treatment was complete.(80-82) Meanwhile Cefaro et al. found no impact on delayed radiation.(83)

In summary the role of radiation timing in local recurrence of early stage breast cancer is inconclusive.

2.3.4.2 Age as a variable

After radiation, young age is the most consistently found variable to be associated with local recurrence in BCT.(64, 84-115) Exactly what age is used to define "young" varies from 32 to 50 with the vast majority of authors using age 35 or 40 as the cut off. Not only is this variable the most consistently found association with local recurrence it is the strongest association next to radiation therapy.

Having said that one study looking specifically at the question of age found no association.(116) They found instead that other factors, such as positive margin, high grade, extensive duct carcinoma in situ (ext. DCIS) and lymphovascular invasion predicted for local recurrence better. In fact, in their multiple variable regression model age was not an independent predictor at all. Moreover, many of the articles that used multiple variable regression did not find age to be an independent predictive variable.

2.3.4.3 Positive margin as a variable.

A positive surgical resection margin has been strongly associated with local recurrence in BCS in a large number of studies. It is widely accepted that re-excision of a positive margin constitutes the current standard of care.(63, 68, 84, 94, 102, 103, 109, 113, 114, 116-127)

More recent literature makes a distinction between focally positive and widely positive surgical resection margins, with some studies demonstrating no difference in local recurrence between focally positive lumpectomies and more widely excised malignancies, especially in the setting of a boost of radiation to the tumor bed.

2.3.4.4 Tumor size as a variable

Early stage breast cancers include both T1 and T2 tumors. The American Joint Committee on Cancer (AJCC) staging manual seventh edition defines T1 tumors as being less than or equal to 2 cm. in size while T2 tumors are "more than 2 cm. but not more than 5 cm. in greatest dimension". Tumor size greater than 2 cm. was one of three variables associated with local recurrence in the NSABP-06 trial (radiation and poor histologic type being the other two). This association has been confirmed in several other studies but it remains one of the weaker associations.(18, 64, 94, 96, 98, 106, 108, 113, 128)

2.3.4.5 Extensive duct carcinoma in situ (Ext DCIS) as a variable

Ext DCIS is defined as being present when 25% or more of the area encompassed by invasive tumor and DCIS is DCIS. There has been a fair body of literature addressing the association between Ext DCIS and local recurrence.(46, 88, 89, 99, 101, 115, 116, 118, 120, 122, 123, 129, 130) While many studies did demonstrate an association with extensive DCIS and local recurrence the significance of this variable is likely diminished or even eliminated if complete pathologic excision can be achieved.

2.3.4.6 Lymphovascular invasion as a variable.

Angiolymphovascular invasion, defined as invasive cancer observed infiltrating lymphovascular spaces is one of the stronger variables associated with local recurrence in BCT.(89, 101, 103, 108, 109, 111, 116, 131)

2.3.4.7 Lymph node status as a variable

Despite a strong association between axillary lymph node status and survival the association with local recurrence in BCT, while present, is not as strong or as consistent.(89, 98, 101, 108, 115, 129, 132)

2.3.4.8 Estrogen Receptor (ER) status as a variable.

As is the case with lymph node status the strong association between ER status and survival does not seem to translate to local recurrence. There have only been a few studies showing an association between ER status and local recurrence in BCS. (89, 101, 111)

2.3.4.9 Grade as a variable

Tumor grade has only variably been associated with local recurrence in BCS.(116, 123, 131, 132)

2.3.4.10 Systemic adjuvant therapy as a variable

Systemic adjuvant therapy was not noted to be an association in the earlier literature on BCT primarily because during that time only node positive women were receiving system adjuvant therapy. Only when more and more node negative women began to receive systemic adjuvant therapy was the possibility of this association considered. It is now widely acknowledged that there is an association with systemic adjuvant therapy (either hormonal therapy or chemotherapy) and decreased local recurrence.(62, 68, 92, 97, 98, 113, 133-136)

2.3.4.11 Other histological types as a variable

The World Health Organization classification of breast carcinoma is as follows: (137)

1. Carcinoma in situ
    a. duct carcinoma in situ (DCIS)
    b. lobular carcinoma in situ (LCIS)
2. Invasive ductal carcinoma
    a. microinvasive carcinoma
    b. invasive duct carcinoma not otherwise specified (NOS) (80%)
        i. Mixed type carcinoma
        ii. Pleomorphic carcinoma
        iii. Carcinoma with osteoclastic giant cells
        iv. Carcinoma with choriocarcinoma features
        v. Carcinoma with melanotic features
    c. Invasive lobular carcinoma (10%)

        The histopathologic features of this cancer include small cells with rounded nuclei, inconspicuous nucleoli, and scant cytoplasm. This cancer is frequently multifocal, multicentric and bilateral.(138-140)
    d. Tubular carcinoma (2%)

        This is another good prognosis carcinoma compared with infiltrating duct carcinoma. Some have suggested that adjuvant radiation could be omitted in these patients because of this.(141, 142)
    e. Invasive cribriform carcinoma

f. Medullary carcinoma (4%)

This type of carcinoma is frequently seen in BRCA-1 hereditary breast cancer. Bilaterality is reported in 20% of cases. It is characterized by a dense lymphoreticular infiltrate composed predominately of lymphocytes and plasma cells, large pleomorphic nuclei that are poorly differentiated and show active mitosis and a sheet-like growth pattern with minimal or absent ductal or alveolar differentiation. Women with this cancer have a better 5 year survival rate.

g. Mucinous carcinoma and other tumors with abundant mucin (2%)

i. Mucinous carcinoma

This tumor typically presents in elderly patients as a bulky tumor. It is defined by extracellular pools of mucin, which surround aggregates of low-grade cancer cells. This tumor has a better prognosis.(138)

ii. Cystadenocarcinoma and columnar cell mucinous carcinoma

iii. Signet ring cell carcinoma

h. Neuroendocrine tumors

i. Solid neuroendocrine carcinoma

ii. Atypical carcinoid tumor

iii. Small cell/oat cell carcinoma

iv. Large cell neuroendocrine carcinoma

i. Invasive papillary carcinoma (2%)

This cancer tends to present later in life and occurs in a disproportionate number of nonwhite women. These tumors tend to be small (rarely greater than 3 cm). This carcinoma has a better prognosis than infiltrating duct carcinoma.(138)

j. Invasive micropapillary carcinoma

This cancer, characterized by delicate pseudopapillary structures lacking a fibrovascular core and tubuloalveolar structures freely floating in clear, empty spaces is quite rare and has a highly aggressive behavior.(143, 144)

k. Apocrine Carcinoma

l. Metaplastic carcinomas

i. Pure epithelial metaplastic carcinomas

Squamous cell carcinoma

Adenocarcinoma with spindle cell metaplasia

Mucoepidermoid carcinoma

ii. Mixed epithelial/mesenchymal metaplastic carcinomas

m. Lipid-rich carcinoma

n. Secretory carcinoma

o. Oncocytic carcinoma

p. Adenoid cystic carcinoma

q. Acinic cell carcinoma

r. Glycogen-rich carcinoma

s. Sebaceous carcinoma

t. Inflammatory carcinoma: defined clinically as enlarged erythematous breast.

In Fisher's NSABP-06 trial tumors of poor histological type were one of the variables identified that predicted local recurrence.

2.3.4.12 Other variables in the literature.

Other variables that have been examined include multifocality, menopausal status and a number of biomarkers including Bcl-2, Ki-67, c-erbB-2, waf-1and p53 but there are few studies that examined these issues.

2.3.4.13 Narrow margin as a variable.

2.3.4.13.1 Overview

Given that this is the variable of primary interest in this thesis all of the papers published in the literature to date that have examined the issue of narrow surgical resection margin were reviewed. A total of twenty-two publications were identified—four that found a positive association and eighteen that did not. Of these twenty-two publications four of them (two showing an association and two not) were the same data sets reported upon at different dates.

2.3.4.13.2 Schnitt SJ et al. The Relationship between Microscopic Margins of Resection and the Risk of Local recurrence in Patients with Breast Cancer Treated with Breast Conserving Surgery and Radiation Therapy.(118)

This retrospective study conducted on 181 patients who underwent BCT was published in 1994. All patients received a higher dose of radiation than is standard (60Gy or higher). A positive surgical resection margin was defined as tumor at the inked margin and was further divided into focally positive and positive, a close margin was tumor within 1 mm, and a negative margin was defined as no tumor within 1 mm of the inked margin. Medium follow up was 86 months. Local recurrence rates were 21%, 6%, 4% and 0% for positive, focally positive, close and negative surgical resection margins respectively. The authors concluded that there was no difference in local recurrence rates based on margin of resection.

Critique:

Like many of these studies this study had too small a number of narrow margin cases to address the issue it purports to. There were only 25 close margin cases, 48 focally positive cases and 38 cases that were more than focally positive cases. They included nodal failure in their local recurrence numbers.

2.3.4.13.3 Gage, I et al. Pathologic Margin Involvement and the Risk of Recurrence in Patients Treated with Breast Conserving Therapy(145)

This retrospective study published in 1996 examines the question of local recurrence in BCS with a particular view to clarifying the Ext DCIS issue. Of 1790 women treated with BCT the 343 with Ext DCIS formed the basis for their study. Surgical resection margins were classified as negative if it was greater than 1 mm from the inked margin, close if it was within 1mm and positive if there was tumor at the inked margin. The positive margins were further divided into focally positive or widely positive. The 5 year ipsilateral breast recurrence rate was 2% for close and 3% for negative margins. Table 2.2 looks at their data by Ext DCIS status. The authors conclude that focally positive tumors could be treated with BCT

Table 2.2 Gage study recurrence by Ext DCIS and margin status

Margin	**Ext DCIS**	**No Ext DCIS**	**Overall**
Negative	2/14(14%)	1/93(1%)	3/107 (3%)
Close	0/10(0%)	1/44(2%)	1/54 (2%)
Focally positive	1/14(7%)	6/65(9%)	7/79 (9%)
Positive	8/19 (42%)	6/31(19%)	14/50 (28%)

Critique:

This retrospective paper has too few narrow margin cases to make a valuable contribution on the question of local recurrence in BCT either for narrow margins or for the question of Ext DCIS.

2.3.4.13.4 Touboul E et al. Local Recurrences and Distant Metastases after Breast Conserving Surgery and Radiation Therapy for Early Breast Cancer(99)

This retrospective study, published in 1999, is a study of 528 patients who underwent BCS. It was designed to look for factors predictive of local recurrence. All histological types were included. A boost dose of radiation was given in about half of the patients. Margin status was classified positive in 13 cases, close (less than or equal to 2 mm) in 21 cases, negative (greater than 2mm) in 417 cases and indeterminate in 77 cases. Multivariate analysis showed only four factor to contribute to the prediction of local recurrence—young age (less than or equal to 40), premenopausal status, bifocality and extensive DCIS.

Critique:

The number of close margins was too small to make use of this study to comment on the contribution of surgical resection margins on local recurrence in early stage breast cancer. (21)

2.3.4.13.5 Freedman G et al. Patients with early stage invasive cancer with close or positive margins treated with conservative surgery and radiation have an increased risk of breast recurrence that is delayed by adjuvant systemic therapy.(146)

This retrospective review of 1,262 stage I and II breast cancer patients seeks to clarify the width of the resection margin that minimizes the risk of local recurrence in BCT. Published in 1999 the study had a median follow-up is 6.3 years and a range of 0.1-15.6 years. Margin status was classified as negative, close (less than or equal to 2mm), or positive. 59% had a re-excision. There were 968 negative margin, 142 narrow margin and 152 positive margin cases at the time of the initial excision. It is unclear how many patients remained in these categories following re-

excision. At 5 years follow up the local recurrence rates between the groups was not statistically different but by 10 years there was a statistically significant difference between the negative group (7%) and either the narrow margin group (14%) or the positive margin group (12%).

Critique:

There are several methodological problems with this study that make it difficult to interpret. Like so many studies examining the issue of narrow margins in local recurrence of early stage breast cancer treated with BCS the numbers are small. They do not tell us how many narrow margin patients there were in this group following re-excision but we do know that the initial size was 142 and that 43% of that group had a re-excision. It is assumed that the size of the remaining group was around 81 (142 x .57). They did not use multiple variable regression techniques so none of the other potential variables were considered in the analysis, including the fact that the narrow margin group had a significant greater number of lymph nodes positive than the wider margin group. Finally, the difference didn't appear until 10 years. There would have been very few patients remaining at that point as the follow-up median was only 6.3 years with a range of 0.1-15.6. For all of the above reasons this study, while adding to the literature, can not stand alone in informing the debate around narrow margins and local recurrence in early stage breast cancer treated with BCS. In fact, the methodological concerns make one wary of drawing any strong conclusions.

2.3.4.13.6 Peterson ME et al Outcomes in breast cancer patients relative to margin status after treatment with breast conserving surgery and radiation therapy: the University of Pennsylvania experience.(147)

This retrospective study of 1,021 early stage breast cancer patients treated with BCT was published in 1999. It was designed to look at local recurrence in relation to margin status. The authors divided the patients into four margin categories based on the final margin status. There

were 518 negative margin patients, 124 focally positive margin patients, 96 patients with a close margin (less than or equal to 2 mm), and 283 patients with unknown margins. The local recurrence rates at 8 years were 8% vs. 10% vs. 17% vs. 16% for negative, focally positive, close and unknown margins respectively. This was not statistically significant. The authors concluded that close margins did not require re-excision.

Critique:

This rather straightforward retrospective study is still underpowered but does add to the debate regarding narrow margins in BCT in so much as it addresses the topic without a lot of extraneous information and can be compared with other studies.

2.3.4.13.7 Park CC. et al. Outcome at 8 Years After Breast Conserving Surgery and Radiation Therapy for Invasive Brest Cancer: Influence of Marin Status and Systemic Therapy on Local Recurrence(148)

This is a retrospective review of 533 patients that was designed specifically to assess the influence of margin status on local recurrence. Published in 2000, it found no difference between patients with a negative or close (less than 1 mm) surgical resection margin (both 7%). Focally positive and extensively positive resection margins had a 14 and 27% local recurrence rate respectively. All cases were pathologically reviewed. Local recurrence included all ipsilateral breast and skin recurrences.

Critique:

While the number of cases (94 close margin, 204 negative, 66 extremely positive and 122 focally positive) was more than most studies it is still under powered. The definition of local recurrence includes both true recurrences as well as new primaries.

2.3.4.14.8 Perez C. Conservation therapy in T1-T2 breast cancer: Past, current issues and future challenges and opportunities(97)

This retrospective study published in 2003 examined local recurrence in BCT in relation to both tumor size and margin status. They found no statistical difference between the groups in local recurrence. Table 2.3 shows their data. They had a total of 1037 patients. A close margin was defined as less than or equal to 3mm.

Critique:

This study has too few close margins for any strong conclusion but showed no statistical difference in local recurrence between the margin groupings.

Table 2.3 Data from Perez regarding local recurrence and margin status.

Size	Positive	Close	Negative	Unknown
T1	1/30 (3.3%)	0/40 (0%)	16/438 (3.6%)	18/196 (9%)
T2	2/16 (12.5)	1/16 (6%)	7/105 (6.6%)	4/68 (5.9%)
Total	3/46 (6.5%)	1/56 (1.8%)	23/543 (4.2%)	22/264 (8.3%)

2.3.4.13.9 Fredriksson et al. Risk factors for local recurrence after breast conserving surgery(149)

This article, published in 2003, was a case-control study. They used a cohort of 7502 women who underwent BCS for either invasive carcinoma or insitu carcinoma. 491 cases of local recurrence were matched with 1098 controls from the cohort. Local recurrence was defined as any histology in the recurrent breast including skin. Axillary recurrences were not considered local recurrences. All pathology slides were reviewed. There were four margin groups. A negative margin was defined a greater than or equal to 1 mm, a positive margin as less than 1mm, a doubtful margin as “close but not measured” and an unknown group that was not further

defined. Multivariate conditional logistic regression analysis showed that age less than 40, radiotherapy, adjuvant hormonal therapy, tumor multicentricity and unclear or unknown surgical resection margins were predictive of local recurrence.

Critique:

It is very unfortunate that this otherwise quite well constructed retrospective case-control study does not shed much light on the question of the significance of narrow resection margins in predicting local recurrence. Especially given that all of the pathology slides were reviewed it is disappointing that we do not know what either an unclear or an unknown surgical resection margin means. Either could be a positive margin, a narrow margin or a combination of both. This reality leaves this study uninterpretable with regards to margin status.

2.3.4.13.10 Smitt, MC et al. Predictors of re-excision finding and recurrence after breast conservation.(150)

This 2003 retrospective review is an update of a previous publication. 535 patients who underwent BCT followed for a mean time period of 6 years were examined for prognostic factors. Margin status was classified as negative, close (less than or equal to 2 mm), positive or indeterminate. Margin status was the most important predictor of local recurrence in their cohort. The results are found in table 2.4.

Table 2.4 Smitt data for local recurrence rates by final margin status

Margin	**Patient number**	**Recurrence in % 6 year actuarial**	**% Crude Recurrence**
Negative	342	3	3
Close	55	22	13
Positive	28	17	18
Indeterminate	110	15	17

Critique:

This retrospective study shows margin status, including narrow margin, to be a significant predictor of local recurrence in BCT for early stage breast cancer. The small number of close and positive margin cases is a concern. With the multitude of other variables thought to have an influence on local recurrence one would need to see these results reported on a more consistent basis to draw cause and effect conclusions.

2.3.4.13.11 Chism DB et al. Re-excision of margins before breast radiation-diagnostic or therapeutic?(151)

This retrospective study, published in 2006, examined only patients with either a close (less than or equal to 2mm) or positive initial surgical resection margin. The 1,044 patients were divided into three groups:

1. no further excision (n=199)

2. re-excision with no residual tumor found (n=546)

3. re-excision with residual tumor found (n=299)

There was no statistical difference in local recurrence between the three groups over a 10 year period.

Critique:

This observational study, which has a reasonable number of narrow margin cases, would suggest that BCS with a narrow resection margin can result in a low local recurrence rate but the lack of comparison to a wider resection margin limits its usefulness.

2.3.4.13.12 Kunos, C et al. Breast Conservation Surgery Achieving >/= 2mm Tumor-Free Margins Results in Decreased Local-Regional Recurrence Rates.(152)

This is a retrospective review of 341 early stage breast cancer patients who were followed for a median of 56 months (range of 10-105 months). Published in 2006 the purpose of the study

was to examine the role of margin status on loco-regional recurrence. Multivariate analysis showed negative ER status, close surgical resection margin and the presence of angiolymphatic invasion to be predictive of local recurrence.

A narrow margin was defined as less than or equal to 2mm. All patients who had an initial narrow margin were re-resected up to two times unless it was deemed that doing so would affect cosmesis. Histologic confirmation of margin status was possible in only 86% of cases. More of the narrow margin group had extDCIS.

The local recurrence rate was 4/22 (1.8%) of the negative group and 10/119 (8.4%) of the narrow margin group. This was statistically significant at p=.007.

Critique:

While this retrospective study does show a statistically significant difference in local recurrence rate between the narrow and wide margin groups there are some methodological concerns other than small sample size that diminish the strength of the study. Local recurrence was defined as any of lymph node recurrence together with both true recurrences and new primaries. There is no evidence in the literature that BCS impacts nodal recurrence in any way. The narrow margin group (which is the smaller of the two) had twice as many nodal recurrences than the wide margin group. Furthermore, by having a policy wherein all patients with a narrow surgical resection margin underwent a re-excision (or two) unless it was deemed to negatively affect the cosmetic result they have inadvertently created a selection bias that would select larger tumors into the narrow margin group as those which could be easily be re-excised would have had a re-excision placing them into the wider margin group. Because of these concerns this study does not contribute significantly to the debate on the significance of narrow resection margins on local recurrence in early stage breast cancer treated with BCT.

2.3.4.13.13 Bollet, MA et al. Age remains the first prognostic factor for loco-regional breast cancer recurrence in young (<40 years) women treated with breast conserving surgery first.(95)

This is a retrospective study published in 2007 that looks at the role of a narrow surgical resection margin in younger aged women. Young age group was focused upon because of the association between young age and local recurrence. Two hundred and nine patients were divided into two margin categories, greater than 3 mm or less than 3 mm. They found that surgical resection margin did not predict for local recurrence.

Critique:

This study was underpowered to resolve the problem with only 209 cases, in total of which 20% or 42 had a close resection margin.

2.3.4.13.14 Vordermark, D et al. Local control in 118 consecutive high-risk breast cancer patients treated with breast conserving therapy.(153)

In this 2007 retrospective review of 118 patients who had close or positive surgical resection margins over a 5 year period the authors conclude that among patients with close or positive margins older patients achieved high local control rates. They noted that younger patients and those who received adjuvant chemotherapy were at increased risk of local recurrence. A close margin was defined as less than or equal to 4mm. There was no statistical difference in local recurrence between the various margin groupings.

Critique:

This retrospective study does not have a wider margin arm and it is significantly underpowered. Their local recurrence rate of 95.5% in 5 years for the narrow margin group is acceptable compared with other studies but their definition of narrow margin (less than or equal to 4 mm) is so different from the rest of the literature that it is impossible to make use of this information.

2.3.4.13.15 Hardy K et al. The Impact of Margin Status on Local Recurrence following Breast Conserving Therapy for Invasive Carcinoma in Manitoba.(154)

This retrospective case control study published in 2008 was designed to shed light specifically on the issue of the contribution of narrow surgical resection margins to local recurrence in early stage breast cancer local recurrence. His group examined all stage I and II breast cancers treated with breast conserving therapy between 1995 and 2004 at the Manitoba Cancer Center.

In a cohort of 3,017 patients treated with BCT in Manitoba 50 cases of local recurrence were matched with 150 controls from the same cohort. These cases were assessed for surgical resection margin and were classified as positive, less than 1mm, less than or equal to 2mm, greater than 2mm, negative, no residual on re-excision or unknown. The mean follow-up was 60 months. They concluded that, "no clear benefit to wider histologically negative margins is demonstrated".

Critique:

Overall this was a well-conducted retrospective case control study that has some limitations worthy of mention. The sample size calculation showed a need for almost three times the caseload as they had. All histologies were included and pathologic review was not used. While it might be reasonable to assume a measured margin to be valid the statement that, "based on informal discussion with local pathologists a report of 'negative NOS' margins was typically used when margins were greater than 2 mm" is not likely a reasonable assumption to make without at least some sort of review to confirm its validity. They identified local recurrences by a couple of different search strategies within the database that was available to them rather than reviewing all of the charts to determine if there was a local recurrence or not. Only cases deemed to be a local recurrence by the above-mentioned process were reviewed. There was no consideration given to other variables known or thought to affect local recurrence.

2.3.4.13.16 Jones, HA et al. Impact of Pathological Characteristics on Local Relapse After Breast Conserving Therapy: A Subgroup Analysis of the EORTC Boost Versus No Boost Trial(155)

This prospective study published in 2009 is a sub-group analysis of the Early Breast Cancer Trialists Collaborative Group (EORTC) Boost Versus No Boost Trial. The main objective of that trial, which accrued 5,569 patients between 1989 and 1996, was to assess the value of a boost of radiation in early-stage breast cancer patients treated with BCT. Margin status was examined in all 1,616 enrolled patients with clinical stage I and II breast cancer for this sub-group analysis. Margin status was defined as positive if invasive or DCIS was seen at the inked resection margin, close if the margin was less than or equal to 2mm, and negative if it was greater than 2 mm. Statistical analysis was with both a univariate and multivariate analysis using Cox proportional hazard models. Local failure was defined as disease recurrence in the treated breast. There were 51 patients with a positive margin, 306 with a close margin and 1,137 with a negative margin. All pathology slides were centrally reviewed. The 10 year cumulative risk of local breast cancer relapse as a first event was not significantly influenced if the margin was scored negative, close or positive. They did find young age and high grade to be important risk factors for local recurrence.

Critique:

This prospective recently performed study is adequately powered and finds no difference in local recurrence based on margin status. Despite the concern that it is a subset of a larger prospective study the information provided by this study should significantly inform the debate around surgical resection margins.

2.3.4.13.17 Perez CA Breast conservation therapy in patients with state T!-T2 breast cancer: current challenges and opportunities(156)

This 2010 publication examines a cohort of 1521 patients treated with BCT examining tumor size and margin status in women under the age of 40. They have a ten year follow up. Table 2.5 shows their data

Table 2.5 Data from Perez regarding local recurrence and margin status in young women

Tumor size	**Negative**	**Close or positive**
T1	9%	12%
T2	12%	22%

Critique:

This is a follow up from their earlier study. There is still no statistical difference in local recurrence between narrow or positive margins (lumped together in this study) and negative surgical resection margins

2.3.4.13.18 Goldstein NS Factors Associated with Ipsilateral Brest Failure and Distant Metastases in Patients with Invasive Breast Carcinoma Treated with Breast Conserving Therapy. A Clinico-pathologic Study of 607 Neoplasms From 583 Patients.(157)

This 2003 publication contains the most extensive pathologic examination of BCS specimens found in the literature. The authors extensively examined slides from 607 consecutive breast carcinomas treated with BCS to see if they could find anything that predicted local recurrence.

All patients with an initial lumpectomy deemed “inadequate” were re-excised as a matter of institutional policy. Recurrence was defined as an ipsilateral breast recurrence. In their series they had 231 narrow margin cases. They created a 5 tiered composite factor of margin distance and amount of carcinoma near the margin such that their close margin group was subdivided into three (close with a least amount of tumor, close with an intermediate amount of tumor and close with the greatest amount of tumor). There were 101, 87 and 43 patients in each of these narrow margin sub-groups. They also subdivided the narrow margins into two groups according to margin (0.1-1.0 mm and 1.1-2.0 mm). Their data is shown in tables 2.6 and 2.7. They found no difference in local recurrence between near and negative margins but a statistically significant increase in local recurrence in patients who had a positive margin. They also found that the amount of carcinoma in near resection margins predicted local recurrence (more so than just a narrow margin).

Table 2.6 Goldstein data local recurrence as a function of final margin status

Final Margin Status	Number of cases	5 year local recurrence rate	12 year local recurrence rate
Unknown	5	10%	2/5 (40%)
Positive	38	12%	9/38 (31%)
Close--total	231	2%	25/231 (12%)
Close 0.1-1.0	94	1%	14%
Close 1.1-2	45	0%	6%
Negative--total	333	3/330 (1%)	30/333 (9%)
Negative 2.1-3.0 mm	59	2%	15%
Negative 3.1-5 mm	43	5%	13%
Negative 5.1-10 mm	90	1%	13%
Negative >10 mm	52	0%	5%

Table 2.7 Goldstein data local recurrence considering amount of carcinoma in narrow margin cases

Amount of carcinoma near the margin	Number of cases	5 year local recurrence rate	12 year local recurrence rate
Least	101	1%	6%
Intermediate	87	3%	18%
Greatest	43	6%	24%

Critique:

This series examines the issue of local recurrence in narrow margin lumpectomy used in BCT from the perspective of the amount of residual tumor at the closest inked margin. The results suggest a kind of dose response curve with the rate of local recurrence increasing with increasing amounts of tumor adjacent to the narrow margin. It is interesting to see that margin status itself made no difference to local recurrence rates in this series. It would have been of value to see if

the volume of tumor at the closest resection margin of the negative lumpectomy specimens correlated in any way with local recurrence but that was not done.

2.3.4.13.19 Karasawa, K et al. Treatment Outcome of Breast Conserving Therapy in Patients with Positive or Close Resection Margins: Japanese Multi Institute Survey for Radiation dose Effect.(96)

This 2005 publication examined a database of 971 women who had a surgical resection margin less than 5 mm. Of this cohort 941 had adequate information and were included in the study. The patients were divided into three groups—positive, narrow (less than or equal to 2 mm) or greater than 2 mm. Local recurrence was defined as an ipsilateral breast recurrence. Their data is presented in Table 2.8. There was no statistically significant difference in local recurrence between the three groups. The radiation dose was less than 60 Gy in 252/941(27%) of the patients, 60 Gy in 456/941(48%) patients and more than 60 Gy in 233/941(25%) patients.

Critique:

This retrospective series has the largest number of narrow margin patients in the literature. The authors show no difference in local recurrence rates out to 10 years. A significant number of their patients had a boost of radiation. Unfortunately this study did not conduct any multivariable analysis. This publication offers a useful contribution to the literature concerning local recurrence following a narrow surgical margin because of the significant number of narrow margins cases.

Table 2.8 Karasawa data regarding local recurrence and surgical resection margin

Margin	Number	5 year local recurrence rate	10 year local recurrence rate
Positive	358	4.9%	14.1%
Narrow	326	3.2%	9%
> 2mm	256	4.8%	13%

2.3.4.13.20 Wazer DE et al. Factors Determining outcome for Breast conserving Irradiation with Margin-Directed Dose Escalation to the Tumor Bed(158)

This paper, published in 1998, studied 509 women treated with BCT with special attention paid to a radiation dose escalation that was used at the authors' institution. Institutional policy was that all patients who had a narrow or positive margin on the initial resection were re-excised if possible. A narrow margin was defined as less than or equal to 2 mm. Their cohort of 509 included 105 positive margin patients and 99 narrow margin patients. Univariate analysis showed no statistical difference in local recurrence between narrow margin resections and wider margin resections. Positive surgical resections were associated with an increased local recurrence. The authors concluded that their dose escalation formula provided an exceptionally low risk of early local recurrence.

Critique:

This paper did not demonstrate any increase in local recurrence in women who had a narrow surgical resection margin. The dose escalation formula used by the authors makes it hard to know how to compare this study to others in the literature.

2.3.4.13.21 Smitt, MC et al. The Importance of the Lumpectomy Surgical Margin Status in Long Term Results of Breast Conservation.(117)

This 1995 publication divided patients treated with BCT into groups based on margin status. A close margin was defined as less than or equal to 2 mm. from the margin. The margin groups were positive, close, negative and indeterminate. Their 5 and 10 year local recurrence rates are shown in table 2.9. Their definition of local recurrence included all ipsilateral breast and skin recurrences. It was unclear if they included nodal recurrences or not. They concluded that "re-

excision appears to convey a local control benefit for those patients with close, indeterminate, or positive initial margins, when final margins are attained".

Table 2.9 Smitt data

Final margin status (n)	**5 year local control (%)**	**10 year local control (%)**
Negative (157)	98	98
Close (17)	84	82
Indeterminate (105)	91	
Focally positive (10)	100	
Diffusely positive (14)	91	

Critique:

There were only a very small number of close surgical resection margins (17 of 303 cases). Unfortunately, they combined the 17 close margin patients with the 105 indeterminate margin patients and the 24 positive margin patients and compared that group with the negative margin group in their analysis rendering the conclusion they made invalid.

2.3.4.13.22 Pittinger, TP et al. Importance of margin status in outcome of breast conserving surgery for carcinoma.(159)

This retrospective study published in 1994 examined 211 women treated with BCT with a view to determine the risk of local recurrence as a function of margin status. In their study a close margin was considered to be less than or equal to 3 mm. They divided the 211 women into four margin groups, negative, close, positive and unknown. Their data is presented in table 2.10. They concluded, "although one fourth of patients with close margins have residual tumor, recurrence rates are similar to those with negative margins".

Table 2.10 Pittinger data

	Negative	Close	Positive	Unknown	Total
Final margin	122	35	4	22	183
Local recurrence (%)	4 (3)	1(3)	1 (25)	2(9)	8(4)

Critique:

This retrospective study has a very small number of close margin patients making their conclusion difficult to accept as definitive.(35)

2.3.4.13.23 Solin, LJ The significance of the pathology margins of the tumor excision on the outcome of patients treated with definitive irradiation for early stage breast cancer. (160)

This retrospective study published in 1991 examined 697 women treated with BCT in early stage breast cancer. These cases were divided into four groups based on the final pathology margin. A negative margin was considered to be one that was greater than 2mm, and a close margin was less than or equal to 2 mm. The two other groupings were unknown and positive. There was no statistically significant difference in local recurrence amongst the various groups. There were 257 negative margin patients, 37 close margin patients, 57 focally positive margin patients and 346 unknown margin patients. Their conclusion was that "selected patients with focally positive or close microscopic pathology margins can be adequately treated with definitive breast irradiation".

Critique:

The small number of narrow margin cases and the large number of unknown margin cases make it difficult to consider this study to be one that significantly contributes to the debate of narrow margin and local recurrence in early stage breast cancer treated with BCT.

2.3.5 Significance of Re-excision

2.3.5.1 Overview

Re-excision of narrow margins would not be much of an issue if there were not negative implications of performing further surgery.(161) Delays incurred as result of a second operation, financial cost, increased risk of wound infection, decreased patient satisfaction with the cosmetic result, and increased anxiety are consequences of re-excision and should be avoided if possible.

2.3.5.2 Financial cost

Every operation has a dollar cost associated with it. Even though the majority of lumpectomies or segmental resections in Canada are performed on a day surgery basis the cost of re-excisions performed for BCT can be significant. In some publications the standard of care was to perform up to 2 additional re-excisions in an attempt to gain a surgical resection margin greater than 2 mm. Upwards of 50% of patients are submitted to re-excision because of either narrow or positive initial lumpectomy resection margin. In a recent series published in 2009 Kouzminova et al. reported results from their institution where the policy was for all patients with a positive or narrow surgical resection margin to undergo re-excision or mastectomy. After the first resection 57 (13%) were found to have a narrow surgical resection margin. Of these 69% required a second operation to gain a negative margin and 8.6% of these required a third operation to clear the margins.(162) With such a policy they would therefore have performed 24 re-excisions for narrow margins for every 100 lumpectomies in order to obtain the negative margins they were looking for. Based on their data 185 re-excisions would be required for every 100 narrow margin lumpectomies to obtain clear margins. Therefore we would anticipate 144 re-excisions annually in Saskatchewan, extrapolating from the database provided for the study done for this thesis, which showed about 600 lumpectomies being performed for breast cancer in

Saskatchewan each year. If one further assumes a cost of around $2,000[1] to perform a re-excision, in Saskatchewan alone the economic burden of that practice would be in the neighborhood of $288,000 annually.

2.3.5.3 Local recurrence risk

The literature examining the issue of whether or not re-excision affects local recurrence in BCT is not consistent. Menes et al. suggested that the risk of local recurrence following BCS is negatively impacted by the need to perform re-excision.(163) O'Sullivan et al. on the other hand found the opposite in their study. (164)

2.3.5.4 Significance of delay to adjuvant therapy

It is self-evident that re-excision would result in a delay in the patient receiving adjuvant therapy. In Saskatoon the time from an initial diagnostic biopsy until a definitive biopsy performed is 28 days. The issues causing that delay are the production of the surgical pathology report and the time to get the patient to the operating room. While that data refers to the time from diagnostic biopsy to definitive surgery it is only reasonable to assume that a similar delay would be encountered with each re-excision as the exact same issues are at play. Re-excision of a lumpectomy is associated with an increase in wound infection, which would potentially delay the start of adjuvant therapy further. A third excision would delay treatment even further. Depending on the amount of delay there could be potential negative repercussions for the individual patient. Delays in receiving radiation are thought to increase the likelihood of local recurrence as noted in the section above and delay in commencing adjuvant chemotherapy may also be associated with increased local recurrence. (74, 120)

[1] Operating room and Pathology cost from the Saskatoon Health Region, physician costs from the Saskatchewan Health payment schedule

2.3.5.5 Psychological impact

Several studies have demonstrated increased psychological distress associated with local recurrence even though there is a greater patient satisfaction with having BCS in the first place.

Saskatchewan's Health Quality Council surveyed women who were diagnosed with breast cancer in 2003 and 2004 about their care experiences.(165) Half (51%) of the 716 women who responded to the survey said they experienced unnecessary anxiety during at least one part of their care. Women felt most anxious during diagnosis and treatment and least anxious during the surgery phase. In addition, nearly half (47%) of women reported feeling out of control during at least one phase of their cancer care. The longest time delay (typically 62 days) occurred between surgery and the start of adjuvant therapy.

The volume of tissue removed with re-excision surgery is more than if only one excision was to be performed. Usually the re-excision requires removal of a significantly greater amount of tissue than an initial wider excision would have, for technical reasons. Studies have shown patient satisfaction to be correlated with the volume of tissue removed.(166, 167)

2.3.6 Significance of a Local Recurrence—Subsequent Treatment

Osteen, Chen and Alpert all conclude that a simple mastectomy is the treatment option of choice in the face of local recurrence following BCT. Selected patients can be eligible for re-excision plus or minus further radiation.(168-170)

2.3.7 Significance of a Local Recurrence—Reduced Survival

As mentioned earlier there is a significant body of literature that supports the safety in terms of survival for BCT. Despite that body of literature studies began to emerge that showed poorer survival outcomes with recurrent tumors. The papers exploring true recurrences versus new primaries make note that true recurrences seem to have a poorer prognosis than the new primaries. How to reconcile these various observations—no difference in survival in patients

treated with BCS and a poorer prognosis in those who do have a local recurrence continues to be debated. Stotter et al. conducted a power analysis and concluded that more than 10,000 patients would need to be randomized to a study comparing modified radical mastectomy with BCT for a minimum of 10 years.(171) A meta-analysis of 78 randomized treatment comparisons of 42,000 women with a 15 year follow up was conducted by Early Breast Cancer Trialists' Collaborative Group (EBCTCG) in 2005.(70) They concluded that "differences in local treatment that substantially affect local recurrence rates would, in the hypothetical absence of any other causes of death, avoid about one breast cancer death over the next 15 years for every four local recurrences avoided, and should reduce 15-year overall mortality". A close examination of this review shows quite a range of studies that make it difficult to draw definitive conclusions. The conclusion the authors make regarding the benefits of improving survival are based on a mathematical model in which it is unclear what they mean by "local treatment that substantially affect local recurrence rates." Moreover they choose to ignore the potential negative effects of radiation on survival. In an editorial arguing for a modification of Fisher's alternative hypothesis theoretical framework Rabinovitch and Kavanagh assert that an earlier meta-analysis by the same EBCTCG shows "there was a highly significant reduction in the annual breast cancer mortality rate for patients treated with radiotherapy after lumpectomy versus lumpectomy alone."(172) In a rebuttal Fisher and Anderson in turn point out that the EBCTCG meta-analysis in fact showed both beneficial and negative effects of radiation with no statistically significant overall effect (table 2.11).(17)

Table 2.11 EBCTCG absolute effect of radiation therapy on survival at 20 years

Deaths	No radiation	Radiation	Difference
Breast cancer related	48.6%	53.4%	4.8 X increase
Non breast cancer related	73.8%	69.5%	4.3X decrease
Overall	35.9%	37.1%	1.2 X increase

While both sides of this discussion clearly feel that they are correct in their understanding of the literature the reality is that the debate is not over. In the absence of conclusive evidence the potential that those women who sustain a local recurrence following BCT for early stage breast cancer having a poorer survival compared to the group who do not, needs to be considered.

2.3.8 Issues Around Accuracy of Surgical Margins

There are a number of problems inherent with determining a surgical margin.(124-126) Balch et al. reported on their institution's experience with using gross margin assessment at the time of the initial surgery by the surgeon and pathologist. They concluded that "gross examination of the resection specimen does not reflect margin status in at least 25 per cent of women undergoing partial mastectomy for breast malignancy."(173) The common manner in which a pathologist determines whether a margin is positive or not is to ink the fresh breast specimen prior to fixing the tissue in formalin. At the time when the data for this study was collected however the most common practice was to fix the specimen in formalin first and then to ink the fixed specimen later. In an editorial addressing this issue Fisher concludes, "it is self-evident that assessment of margins of resection by extant methods is a limited procedure."(174) Given the nature of breast tissue (primarily fat), the ink tends to seep into the cracks and crevices of the fat and can give the impression of a margin that is closer than is real. While this has been addressed as a concern there was no study found that directly looked at this question. Many of the breast specimens

obtained today are harvested using a wire-guided technique because they are not palpable. In those specimens the sample is checked in the radiology department to be certain that the area of concern has in fact been removed prior to sending the specimen to the pathologist. During the time period when the retrospective review was conducted for this thesis the specimens were routinely compressed in a device especially created for this purpose (in order to get an optimal radiographic image). The process of compressing the fresh resected specimen risks fracturing the fat allowing for ink to creep closer to the tumor.(175) Current practice no longer requires specimen compression and the fresh tissue is inked and cut prior to fixation. Finally, the process of fixing tissue in formalin results in shrinkage, with the end result that the measured margin does not reflect the margin achieved at the time of surgery. There have been several studies demonstrating the importance of this issue in tissue with high elasticity but no study was found that examined this issue in breast specimens.

In an attempt to minimize these concerns a variety of techniques have been advocated to try and optimize the accuracy of assessing surgical resection margins. Some authors have advocated the examination of "shaved" margins.(176-178) This has had variable results and has not evolved to become a standard of care partly because of concerns regarding accuracy and partly due to the inherent additional cost. Some authors have advocated frozen section assessment but others feel that this technique compromises definitive assessment and should be avoided.(179-181) Likewise cytological examination of surgical margins has been tried and found wanting as has the use of monoclonal antibodies.(182, 183) Radiographic assessments of surgical margins as well as gross surgical evaluation have been shown not to be reliable.(184)

To complicate matters more not all margins carry the same significance. A narrow surgical resection margin for a tumor that is close to the skin, especially if an ellipse of skin is resected

along with the tumor, has little significance as there is no further margin to be had other than air. Likewise a close margin posteriorly has questionable importance as the pectoralis major fascia acts to some extent as a barrier and standard surgical practice would not include resecting the underlying pectoralis major muscle in the absence of clinical invasion.(185-187) Tumors that are close to these margins do not have the same implication for residual disease as tumors approaching a margin where residual breast parenchyma remains.

Finally the issue of specimen orientation is one that continues to vex the pathologist today. Even with current methods aimed to orient the pathologist it can be a difficult task to fully translate exactly where the specimen came from in the three dimensional breast making identification of the exact location of a positive or close surgical resection margin challenging at best.

2.4 Review of the Methods Used for Prognostication and Prediction in Breast Cancer

2.4.1 Clinical Grading System

Early on in the history of breast cancer treatment there was an awareness that breast cancer is not one disease but in some way a heterogeneous collection of diseases with differing clinical courses. It is possible to see one person with widespread metastatic disease and a primary in the breast that is barely detectable (or not at all) and the same day see another person with a large advanced tumor and no discernable metastasis.

In our collective efforts to attempt to understand and treat this mix of disease called breast cancer a number of classification tools have been developed to aid in the process of prognostication and prediction of treatment response. The beginning of these efforts was the histologic grading system that continues to be the backbone of our current clinico-pathologic system. Most recently genetic profiling has offered new opportunities and has generated a

significant amount of excitement and enthusiasm, rekindling the hope of one day being able to find a way to truly "personalize" the delivery of care to each individual patient.

The clinical grading system that is widely in use today, and which is the standard in Saskatoon, is the Nottingham combined histologic grading system (or the Elston-Ellis modification of Scarff-Bloom-Richardson grading system).(188) This grading system takes into account three different histologic characteristics and scores each one from 1-3 to give a final score out of 9. The grading system examines the following three areas:

1. Tubule formation:

If > 75% of cells arranged in tubules, then score 1.

If 10-75% of cells arranged in tubules, then score 2.

If < 10% then score 3.

2. Number of mitoses:

The mitotic score is determined by the number of mitotic figure found in 10 consecutive high-power fields in the most mitotically active part of the tumor and using a table that takes into account the field diameter and area as these vary from one microscope to another. The score ranges from 1-3.

3. Nuclear pleomorphism:

If cell nuclei are uniform in size and shape, relatively small, have dispersed chromatin patterns, and are without nucleoli, then score 1.

If cell nuclei are somewhat pleomorphic, have nucleoli, and are of intermediate size, then score 2.

If cell nuclei are relatively large, have prominent nucleoli or multiple nucleoli, coarse chromatin patterns, and vary in size and shape, then score 3.

A final combined Bloom-Richardson (B-R) grade is determined by adding the scores of the three areas being examined giving a possible range of 3-9. A modified Bloom Richardson score of 3,4,5 is considered to be a low grade tumor, a score of 6 or 7 results in an intermediate grade designation and a score of 8 or 9 results in a tumor being called high grade.

While there has been concern in the literature about the reproducibility of this grading system two well done studies show there to be quite good reproducibility between pathologists examining the same malignancy.(189, 190, 191) Various studies have demonstrated that histologic grade is a powerful prognostic parameter despite some variation in reproducibility mentioned above.(192, 193)

2.4.2 Immunohistochemical Markers

Immunohistochemistry (IHC) is now an accepted component of modern pathology. It refers to the process of detecting antigens in cells of a tissue section by using tagged antibodies that bind specifically to antigens in biological tissues and allow the pathologist to visualize or see the areas that are bound. IHC is most frequently used in anatomic pathology to help determine an undifferentiated cell's tissue of origin or distinguish between tumor types (the use of e-cadherin to distinguish duct carcinoma in situ where it stains positively from lobular carcinoma in situ where is stains negatively for example).

The discovery of hormone receptors in breast carcinoma resulted in a significant improvement in our ability to classify breast cancer from both prognostic and treatment perspectives. Both estrogen (ER) and progesterone (PR) receptor levels have been found to be useful and it is currently the standard of care that the estrogen receptor status be measured in all invasive primary breast cancers.(133) ER and, to a lesser extent, PR levels are associated with a good prognosis. They are also predictive of response to endocrine treatment with agents such as tamoxifen, aromatase inhibitors, irreversible ER inhibitors and ovarian ablation.(133)

Despite the longstanding use of ER and PR assays in the care of breast cancer patients there has been a lack of standardization for these assays until recently. While these assays were initially biochemical assays when they were developed in the 1980's, the current gold standard is IHC, performed on formalin-fixed, paraffin-embedded cancer tissue.(194) Unfortunately there is substantial intra, and interlaboratory variation in ER and PR results because of fixation, antigen retrieval, and staining methods that differ among laboratories.(135, 136) In one study comparing 200 laboratories receiving sections from the same three reference tumors the false negative rates were as high as 30-60%.(195) It has been demonstrated that the duration of formalin fixation affects ER staining, with longer fixation times resulting in higher ER levels. Despite this there has been no standardized of fixation in most, if not all, laboratories until recently with the introduction of the College of American Pathologists guidelines in 2009.(196, 264) The method and duration of antigen retrieval also affect IHC results. These various concerns combine to give one pause especially considering that there is some data to suggest that the higher the level of expression of ER, the higher the probability of benefit from endocrine therapy. (197)

There are now several methods that can reliably measure ER and PR miRNA expression. Most notable amongst these is quantitative reverse transcription polymerase chain reaction (RT-PCR) and more recently DNA microarrays.(194) These are thought to be more useful not only because they are more reproducible but also because they also measure the expression of several downstream ER-regulated genes (PR, Bcl-2, SCUBE-2) that may contain information on ER functionality.(198) Current IHC assays give us information on the presence of ER but do not guarantee functional activity of those receptors.

A number of serum tumor markers have been examined to assess their potential in aiding with the diagnosis and/or management of breast carcinoma. To date none of these have been found to

be of enough use to incorporate into current clinical practice.(133) CA15-3, CA27 CA29, and CEA are the ones that have been studied the most.

A number of IHC based markers of proliferation in breast cancer have also been studied and not found useful in clinical practice at this time. The most commonly studied in this group have been Ki 67, TK, cyclin E, cyclin D, cyclin inhibitors p27, p21 and topoisomerase Iia.(133)

Cathespsin D and cyclin E are two other newer assays that have been examined and found to be associated with a poorer prognosis but for whom enough evidence has not been accrued to justify them being used clinically in any way.(133)

p53 as a marker for breast cancer deserves special attention given that it is one of the markers that was tested for in Saskatoon during the time period that was used in this study and given the importance that this gene mutation has. It is known to be the most mutated gene in cancer.(199) A number of studies suggest that high tissue p53 protein levels measured by IHC or PCR (polymerase chain reaction) are a predictor of poor outcomes.(139-142) A meta-analysis done in 1999 suggests that p53 mutations confer an independent relative risk of 1.7 for both disease-free survival and overall survival.(200) IHC for p53 detects both mutated p53 and stabilized wild-type p53 and therefore misses p53 deletions. Consequently, it seems unlikely that IHC for p53 will provide accurate enough results to be clinically useful. This is likely a marker that will be useful in the future as methodologies improve and as further research more clearly defines its role.(133)

Her2 is a member of the epidermal growth factor receptor family (EGFR). It is amplified and over expressed in 15-30% of newly diagnosed breast cancers and is associated with a more aggressive behavior. Its measurement is currently considered to be standard of care for all newly diagnosed breast cancers.(133) Her2 is measured either by IHC or by a gene amplification

process (most commonly by fluorescent in situ hybridization or FISH). While Her2 over expression is generally associated with a poorer prognosis currently the use of Her2for determining prognosis is not recommended. It is however recommended to select patients for anti- Her2 based therapy. (133)

Trastuzumab is a humanized monoclonal antibody that binds to the extracellular domain of Her2. Studies demonstrate that for Her2 positive patients, both metastatic and other wise, trastuzumab in addition to conventional chemotherapy has been beneficial.(133)

Moreover evidence exists which suggests that over expression of Her2 identifies patients who have a greater benefit from anthracycline-based adjuvant therapy compared with CMF.(133) Having said that it is not clear whether Her2 itself is the target of anthracyclines or if Her2 status serves as a surrogate for a different gene product that may be the target of the anthracycline.

2.4.3. Nodal Status

It is well accepted that axillary nodal status is the most significant single factor in patient prognosis with node positive patients fairing significantly worse than node negative patients.(144, 145) Moreover prognosis is inversely proportional to the number of positive nodes.

2.4.4 Scoring Systems

There are several scoring systems currently available that make use of clinical and pathologic indicators to predict prognosis and response to treatment. Adjuvant online makes use of the SEER database in the development of its system which relies on histologic grade, tumor size, the number of lymph nodes involved and the estrogen receptor status.(201) The Nottingham prognostic index is derived from a multivariate analysis of operable breast cancer patients in Nottingham.(202) While these two systems are largely equivalent there are some significant differences with the Adjuvant online system being somewhat more optimistic in general.

While these various systems are helpful in predicting prognosis and response to therapy for the individual patient it is not ideal.(148-150) For example approximately one-third of lymph node-negative patients who are currently classified as within a "good prognostic group" develop recurrence while a similar proportion of node-positive women remain disease free. Likewise a significant proportion of patients deemed to be in a "poor prognostic group" will never develop distant recurrence.(203) This reality has been the impetus behind research looking into alternative ways to achieve this goal.(135, 150)

2.4.5 Gene Profiling

2.4.5.1 Overview

With the mapping of the human genome it became evident that genetic changes were at the heart of cancer development.(134, 135, 150-152) In 1986 the NIH officially launched the pilot stage of an effort to create a comprehensive catalogue of genetic changes involved in cancer (The Cancer Genome Atlas or TCGA). By 2007 350 cancer-related genes were mapped in this ongoing work.

One of the key technical advances that the human genome project created was the development of microarray analysis. A microarray is an orderly arrangement of known or unknown DNA samples attached to a solid support. Each DNA spot on the microarray (called the probe) is usually less than 200μM in diameter and an entire array typically contains thousands of spots.(204) There are many ways this could be done but in practice only a small number of formats are in common use. Of these the most common are filter arrays, glass DNA microarrays and high-density oligonucleotide arrays.(203) Cooper in his review of the subject goes into these in some detail. (204)

Microarray technology provides a method for monitoring the RNA expression levels of many thousands of genes simultaneously in primary tumors and cell lines.(204) This technology has

enabled “discovery-based research” to be conducted, in which large volumes of data are generated from clinical specimens and analyzed without a specific hypothesis, in contrast to the traditional scientific paradigm of “hypothesis-based research” in which a limited number of genes/proteins are investigated based upon a specific hypothesis and rationale.(135, 154) The major challenge has been, and continues to be, distinguishing meaningless mutations in tumor samples from cancer related ones.

Gene expression profiling has been introduced in the last decade as means by which multiple genes in a tumor sample may provide useful information about a given tumor’s behavior.(204) Breast cancer research has been at the forefront of this gene expression profiling research.(155) Gene expression is the term used to describe how active a particular gene is.(205) This is a function of how frequently it is expressed or transcribed to produce the protein it encodes. The first step in the process is the transcription of the gene’s DNA into messenger RNA (miRNA). Modern molecular biological tools measure this activity by counting the number of miRNA molecules in a given cell type or tissue. Counting miRNA transcripts provides an estimate of the number of corresponding proteins because the miRNA molecule is translated within the ribosome to produce a complete protein. DNA microarray and real-time reverse transcriptase polymerase chain reaction technologies allow simultaneous counting of many gene transcriptions. Gene expression profiles or signatures are lists of genes that are differentially expressed between normal and diseased patients.

There are two main types of expression microarrays, cDNA microarrays and oligonucleotide microarrays. Both types are hybridized with cDNA or RNA samples obtained from tissue. Real time PCR or qRT is an important method in the armamentarium of micro array analysis.(206)

This technique is particularly attractive because it can be applied to both fresh tissue and paraffin embedded tissue allowing for review of archival specimens.(156-158)

In principle there are two ways that gene expression microarrays can be performed: unsupervised and supervised. Unsupervised analysis refers to an extensive set of methods of which hierarchical clustering analysis has become the most popular. The main purpose of this method is to identify new groupings of genes that may have clinical significance. The strength of these groupings is that they are not based on single genes or a specific pathway. This constellation of several groups of genes then make up a fingerprint or 'portrait'.(203) Supervised analysis on the other hand requires tumor cases to be allocated to specific groups based on clinical or pathological features such as survival or basal cell morphology. There are in turn two main subtypes of supervised analysis, class comparison and class prediction.

Hierarchical clustering algorithms group samples together based on similarity in their patterns of gene expression. This was the method utilized by the Stanford group in their ground-breaking study examining gene-expression patterns of breast cancer in which at least four major molecular classes of breast cancer were suggested.(155, 159) These investigators applied this class discovery method to data from three normal breast samples and 40 different breast tumors (including 20 repeated measurements from the same tumor). They selected the genes that showed the greatest between-sample variability. Using these 1753 genes in hierarchical clustering they observed two main clusters and additional smaller secondary clusters. This data led to a schema of four different molecular classes of breast cancer that they called luminal-like, basal-like, normal-like and Her2positive. They compared the four molecular classes with the tumors ER status. They found that the ER+ group is characterized by a higher expression of breast luminal epithelial cells ("luminal " cancer). This first group has been subsequently divided into at least

two subgroups—luminal-A and luminal-B. Within the ER- group three of the molecular subgroups dominated—one over expressing Her2, one expressing genes characteristic of breast basal/myoepithelial cells (basal-like cancer) and another with a gene expression profile similar to normal breast tissue.(198)

Since then a number of studies have suggested further sub classification schemes.(160) Rakha et al summarize the currently accepted molecular classes in their review. (203) Luminal tumors (ER-positive tumors) are generally subdivided into Luminal-A and Luminal-B. Luminal-A tumors are ER positive, make up 19-39% of tumors, have the highest expression of the ER and ER-related genes and show the best prognosis. Luminal-B tumors have profiles enriched for 'luminal genes' but show low to moderate expression of genes pertaining to the ER cluster. They represent 10-23% of tumors and are associated with a less favorable outcome. ER negative tumors are further subdivided into three molecular and biologically distinct groups. The first of these, called basal-like, makes up 16-37% of tumors. Tumors from patients carrying BRCA1 mutations fall within the basal-like subgroup. Her2 positive tumors represent 4-10% of tumors. These tumors express high levels of genes located in the Her2 amplican (17q11). Both the basal-like group and the Her2 group exhibit aggressive clinical behavior and poor prognosis. Both tend to have high levels of p53 mutation. Finally the normal breast-like group is found in up to 10% of all breast cancers. These tumors have a prognosis that seems to be better than the basal-like cancers and do not appear to respond to neoadjuvant chemotherapy as well as other ER negative tumors. It is likely that further clinical sub stratification will occur.(134, 145, 160, 161)

At the same time as this work has generated significant enthusiasm there has been a number of voices expressing concern. Hierarchical clustering by its very nature is rather subjective. Clustering algorithms always detect clusters, even in random data. As a consequence it is easy to

misinterpret dendritogram results.(198) McShane et al proposed a two-step approach in which a global test for clustering is performed and when a pattern emerges the strength of cluster-specific reproducibility be assessed. (207) Applying this to the initial Stanford data demonstrated robustness and reproducibility only for the original four classifications. Clustering results also depend on the gene set that is used. It is important to use a standard gene set, otherwise clustering yields different results even for the same data. In addition sample size can significantly alter the results even when the same gene set is used for clustering. Other variables such as data input, choice of distance metric, and linkage can also have profound effects on the shape of the dendritogram generated by hierarchical clustering.

Quite apart from the technical concerns regarding unsupervised gene expression analysis several significant and important questions remain unanswered. Whether or not these molecular subgroups really do represent a new and novel approach and how much if any additional information this classification system offers over traditional methods in terms of patient management is still unclear.(203) One could argue that what we are seeing with hierarchical clustering expression data is merely a reflection of the ER status, the Her2 status and the proliferation status that can be determined using other routinely available techniques. At the end of the day we do not know yet whether or not molecular taxonomy will outperform current classification systems. Moreover molecular classification based on hierarchical clustering analysis cannot be applied prospectively. And finally we need to acknowledge that currently 6-36% of breast cancers cannot be classified into any of the identified categories.

In class comparison studies one compares groups of interest that are not defined by expression profiles. For example invasive versus in-situ cancers might be examined via a given gene expression profile. Rahka et al note that class comparison studies have for the most part

corroborated concepts obtained by means of traditional pathology and molecular genetic studies.(203)

The major aim of class prediction studies is to identify a set of key genes that can accurately predict the class membership of new samples based solely on that predefined predictor set. Within this group of studies there are again two types of studies with different goals. First we can identify a group of studies that use gene signatures to prognosticate and second we can identify a group of studies that use gene signatures to predict response to therapy. This paper will examine in some detail the various significant profiles in some detail.

2.4.5.2 Clinical use

Van't Veer et al pioneered the concept of prognostic class prediction in their study that identified a list of 70 discriminatory genes.(155, 163) The "Amsterdam 70-gene prognostic signature" was found to be strongly predictive of a short interval to distant metastasis in lymph node-negative patients.(164) This 70-gene predictor is commercially available as the "Mammaprint" assay and is being used a clinical trial called MINDACT (Microarray in Node negative Disease my Avoid ChemoTherapy).

In the development of this signature approximately 25000 genes were studied in 97 node-negative breast cancers. A training set of 78 of these 97 tumors (34 from breast cancer patients who developed distant metastases during the 5 years after surgery and 44 from patients who did not) was used to develop the classification rule. A statistic measuring the difference in gene expression between the two types of tumors, with and without metastasis at 5 years, was calculated for each gene. The 70 top-listed genes were selected and their average expression in good prognosis tumors was defined as the good prognosis profile. Each of the remaining 19 tumors from the validation set was classified according to the correlation between its 70 gene expressions and the good prognosis profile.

The Oncotype Dx assay developed and commercialized by Genomic Health was developed to determine the risk of recurrence in women with node-negative, ER+ breast cancer who had received treatment with tamoxifen and may not benefit from adjuvant chemotherapy vs. those for whom CMF adjuvant chemotherapy might be more advantageous than tamoxifen.(134, 151, 165, 166) This assay is done on formalin-fixed paraffin-embedded tissue samples and is based on the expression of 21 genes (16 cancer-related genes and 5 control genes). (208) The 21 genes in Oncotype DX were selected from a much larger set of genes following the analysis of patients enrolled in the NSABP B-20 trial.(209) The levels of expression of the 21 genes are manipulated by an empirically derived prospectively defined mathematical algorithm to calculate a RS (recurrence score). This RS score is then used to assign a patient to one of three groups by estimated risk of distant recurrence: low, intermediate, and high. Once the algorithm was derived it was validated on another group of patients who were enrolled in the NSABP B-14 trial. By multivariate Cox-model analysis the test was a significant predictor of recurrence independent of age and tumor size and a significant predictor of overall survival. A large retrospective set of specimens from Kaiser Permanente with long follow-up was also used in the validation process. It is an RT-PCR assay. A trial assigning individualized options for treatment (TAILORx) is currently undergoing accrual with a view to validate its usefulness as a predictor of chemotherapeutic response in patients with ER+ node-negative tumors.(194, 210)

Of the multiple pathways assessed by the assay, the proliferation and ER pathways are the most influential on the RS calculation followed by the Her2 pathway. It should be noted that this assay is best suited for detecting breast cancers with a low potential for recurrence.

The oncotype DX discovery cohort consisted of 447 stored samples from three sources. The test was then validated on 668 ER-positive, lymph node-negative cases of tamoxifen-only treated

breast cancer patients of various ages who were enrolled in NSABP B-14 trial. In this validation cohort 50% had tumors with a low RS, and 6.8% of these recurred at 10 years. In the high RS group (27% of the cases) 30.5 % recurred at 10 years. A subsequent validation study using the NSABP B-20 patients demonstrated that the assay predicted benefit from tamoxifen in those with a low or intermediate RS and benefit from chemotherapy in those with a high RS.(209) It must be noted that the patients in the tamoxifen treatment arm from that analysis were the same patients from whom the RS was developed.

Oncotype DX, which is approved by the California State Licensing Agency for Laboratories, has not been submitted to the FDA for formal approval. It is currently exempt from the standard review FDA requires for diagnostic kits. The test has therefore been successfully marketed without classic prospective validation.(165)

The "Rotterdam" gene expression test consists of a 76-gene microarray assay that does not overlap with either the Oncotype DX or MammaPrint assay.(194) This assay was the result of studying 286 node negative patients who had not received adjuvant systemic therapy. Validation was performed on 171 different node negative cases. The hazard ratios for distant metastasis-free survival in premenopausal, postmenopausal and subsets of lesions between 1.0 and 2.0 cm were all statistically significant.

The Mammaprint is a commercially available gene expression-profiling platform marketed by Agendia and is often referred to as the "Amsterdam" profile as it was developed there.(134, 151, 165) It requires fresh tissue for analysis. Analyzing primary tumors from 117 node negative breast cancer patients with oligonucleotide microarrays developed this assay. The data were subjected to supervised classification to establish a 70-gene RNA expression profile that correlated with a relatively short interval to distant metastases. The signature was then tested in

295 consecutive stage I or II primary breast cancer patients younger than 53 years of age. (Of note this second group included 61 patients with node negative disease used in the prior study that established the test). The estimated hazard ratio for distant metastases by signature was 5.1 (p=<. 001).

Ma and colleagues developed a "2-gene signature."(134, 151) It is a 6-gene multiplex prognostic RT-PCR assay that uses FFPE tissues. It is based on the original report of the impact of the ratio of the relative miRNA expression of two genes—the homeobox gene-B13 and the interleukin-17B receptor gene—to predict recurrence in patients with ER-positive, node-negative primary breast cancer.

2.4.5.3 Concerns

Koscielny in his critical review of microarray-based prognostic tests provides an excellent review of the criticism of the current micro-array research. (211) His concerns are as follows:

Gene lists are unstable. Taking the Amsterdam signature as an example he aptly points out that there is no justification for choosing particular tumors for the training and validation sets. They reanalyzed the original Amsterdam data at random such that different tumors were represented in the training and validation sets. They discovered that every set of patients led to a different list of genes in the signature. Ein-Dor estimated that several thousand biological samples would be needed to obtain a stable list of genes.(212)

Validation of the prediction rule. Again using the Amsterdam signature as an example he points out that the validation population included 61 patients who were already in the first study, essentially invalidating the conclusions.

The superiority of signatures over clinico-pathological parameters is not established. Using multivariate regression analysis as is frequently done is poor model building. He argues that the common practice of starting with a signature that is a significant predictor of outcome and then

testing to see if the addition of clinico-pathologic factors improves the model is incorrect. The correct model building strategy would be to comparing two models with and without the signature. When the later was done it was shown that the gene signature did not significantly improve the predictive accuracy of age, nodal involvement, ER status and tumor grade.

In breast cancer, the available signatures lead to discordant predictions. Fan et al applied five gene-expression-based models to a single data set of 295 samples. (213) The five models used were intrinsic subtypes (Stanford group molecular classification system), 70-gene profile (Amsterdam model), wound response, recurrence score (oncotype dx) and the two-gene ratio system. Despite the fact that these various models used very distinct gene sets with little overlap in terms of gene identity, there was a high rate of concordance in their outcome predictions for the individual samples (with the one exception of the two-gene model). This would suggest that these various models are probably tracking a common set of biological phenotypes. Koscielny however points out that among the patients with an intermediate risk according to the recurrence score about half of them are classified as poor-prognosis patients according to the 70-gene signature. He goes on to state that this 50% discordance is clearly not clinically helpful.(211)

Most of the existing studies are significantly underpowered and the conclusions are not supported by the data. The fact that a gene belongs to a given signature does not make it, per se, a functionally and biologically relevant gene for breast cancer. Studies have suffered from bias in sample selection, in statistical analysis and in the analysis of data based on preconceptions of outcome. Some signatures have been found not to be reproducible in independent studies.(194) Multiple signatures have been shown to have prognostic significance in the same cohort of patients. Intra- and interlaboratory reproducibility of results must be determined, and the standardization of methodology has to be established.(134, 135, 151) As a result of these

concerns the US Food and drug Administration has launched the Microarray Quality Control project but the result of this inquiry is still pending.(214)

2.4.5.4 Evidence to suggest use of gene signatures in predicting local recurrence.

There is little evidence in the literature examining the utility of gene signatures in predicting local recurrence following breast conserving surgery.(166, 170) Oncotype DX has been demonstrated in one unpublished report to predict the risk of loco-regional recurrence in node-negative, hormone-positive women with breast cancer. The study presented at the 28th Annual San Antonio Breast Cancer Symposium was an evaluation of the results from the previous NSABP B-14 and B-20 trials in an attempt to determine whether the recurrence score from Oncotype DX could establish an association between loco-regional recurrence in women. They found that the recurrence score was a significant predictor of loco-regional relapse among the 895 tamoxifen-treated patients who were lymph node-negative, estrogen receptor-positive (8 at 10 years the local recurrence rates were 4.3%, 7.2% and 15% respectively for those with low, intermediate and high recurrence scores). The recurrence score also significantly predicted risk of local-regional relapse in placebo-treated patients (20% for intermediate and high recurrence score patients and 11% for those with a low recurrence score.

Kreike et al looked at gene expression profiles in patients under 51 who had undergone breast conserving surgery.(171) They applied various methods of supervised and unsupervised analysis and found no signature that was predictive if ER-driven genes were taken out.

Nuyten et al found that a supervised/optimized wound signature was found to be a robust predictor of local recurrence after breast conserving surgery.(172) In a multivariate model, including clinical and pathological variables, the wound signature was not only independent in predicting local recurrence, but it was also the strongest predictor overall. This observation needs to be verified.

2.4.6 MicroRNA

MicroRNA (miRNA) are a relatively newly discovered class of single stranded non-coding RNA molecules that are 17-27 nucleotides in length.(173, 174) First identified in 1993 these small molecules have been identified in many organisms including yeast, fruitflies, plants and of course humans.(215, 216) Bartel identified that these small miRNA play key roles in regulating gene expression by inhibiting translation and/or triggering degradation of their target miRNA.(216) He demonstrated that RNA Pol II generally transcribes miRNA genes in the nucleus and gives rise to a larger primary miRNA transcript that are processed by RNase III Drosha to form a somewhat smaller pri-miRNA which in turn is transported into the cytoplasm of the cell. Once in the cytoplasm it is cleaved into an imperfect double stranded RNA termed miRNA/miRNA. This miRNA duplex is subsequently unwound and a mature miRNA ultimately binds to miRNA 3' untranslated regions. The two proposed mechanisms of expression from this point related to the degree of complementarities between the miRNA and its target miRNA. By binding to imperfect complementary sites of the target miRNA the miRNA interferes with protein synthesis. The second mechanism is thought to be due to endonucleolytic cleavage of the target miRNA by perfect base pairing, a process thought to function only in flowering plants until recently.

It is estimated that the human genome contains about 1000 miRNA.(217) These miRNA in turn target thousands of miRNA affecting all cellular processes. They are often organized in tandem and found clustered on the chromosome. Over half of the miRNA are located in or near fragile sites or cancer associated genomic regions.(177) Calin et al reported the first link between miRNA and cancer in 2002.(178) Visone and Crose point out that miRNA expression can be altered by several mechanisms in human cancer including chromosomal abnormalities, epigenetic changes, mutations and polymorphisms (SNPs), and defects in the miRNA biogenesis

machinery.(218) All tumors investigated to date have demonstrated altered miRNA expression. It is thought that this may likely implicates miRNA in oncogenesis. This process is likely via the regulations of oncogenes and tumor suppressor genes, cell cycle regulation, regulation of cancer associated signaling pathways and/or the regulation of progression and metastasis.(174, 179-182)

The potential application of miRNA research includes areas of diagnosis and prognostication and potentially as a therapeutic modality.(218) MiRNA expression profiling using techniques similar to those described for DNA profiling above have shown that miRNA clusters samples around the tissue of origin making it a powerful tool in sorting out metastatic tumors of unknown origin. A number of studies suggest a role for specific miRNAs in identifying disease progression and identifying poor prognosis tumors.

Recent studies have shown that miRNA expression profiling may be more accurate for distinguishing disease states than miRNA expression analysis. (183-185) This is because miRNAs are better preserved, and can be obtained from formalin fixed paraffin embedded (FFPE) tissue. As a result it is thought that they may be a better choice for expression studies when using FFPE samples.(219) There are vast collections of FFPE samples of tumors available throughout the world as this was the method of choice worldwide for the preservation of tissue samples for decades now because this method maintains morphological features of the original tissue quite well. These collections of FFPE samples offer the potential for extensive retrospective analyses with significant periods of clinico-pathological follow-up provided the molecular information of interest is available. Unfortunately, the processes of fixation, embedding and storage have negative impacts on the quality of DNA and RNA that are isolated from these tissue samples.(219) Until quite recently there have been no pathology standards regarding length of time in fixation and other details such as the amount of fixative required and

the strength of the fixative. At a molecular level the important issues are nucleic acid fragmentation and modification. Formaldehyde causes crosslinking with proteins and other proteins leading to a significant reduction in the RNA recovered.(220) Moreover, Formalin fixation and ethanol processing also leads to the production of mono-methylol and ethoxylated adducts with the bases of nucleic acids, as well as depurination fragments, reducing the efficiency of reverse transcription.(186-188) RNA fragmentation occurs for several reasons. Delays that occur in getting a sample into formaldehyde result in tissue autolysis and degradation of RNA.(221) Likewise prolonged fixation (more than 24 hours), incubation at elevated temperatures during the embedding process and prolonged storage of the samples (greater than one year and especially at higher temperatures) results in RNA fragmentation.(219) Because of their small size and possibly because of a protective protein coat miRNA's are less susceptible to these effects and are more useful for expression profiling in FFPE samples that have been stored for a period of time.(174, 190)

Aberrant expressions of miRNA were first reported in breast cancer by Iorio et al. in 2005.(222) The identified 29 aberrantly expressed miRNA in their analysis of 79 breast cancers and 14 human breast cancer cell lines. Many of these aberrant miRNA were located at genomic fragile sites or regions associated with cancers.(223) Subsequently Zhang et al. also reported aberrant miRNA in human breast cancers.(224) The miRNA that are associated with breast cancer can be grouped into tumor suppressor miRNA and oncogenic miRNA. The tumor suppressor miRNA identified so far include Let-7 which targets Ras, and Hmga2, miRNA125a/b targeting ErBb2 and ErBb3, miRNA206 targeting ERa, miRNA17/20 targeting AlB1, Cyclin D and E2F, miRNA145 targeting RTNK, and miRNA205 targeting the HER3 receptor. The

oncogenic miRNA identified to date include miRNA21 targeting Tpm1 and PDCD4 and miRNA27 targeting ZBTB10 and Myt 1.

The miRNA let-7 is associated with breast cancer stem cells. It is reduced in these stem cells and increased with differentiation, implying that let-7 can regulate self-renewal and tumorigenicity of breast cancer cells. Let-7 over expression in breast cancer stem cells inhibited cell proliferation and mammosphere formation in NOD/SCID mice.

CHAPTER 3
METHODOLOGY

3.1 Introduction

This chapter will provide the reader with a detailed explanation of the methodology employed for the study that was performed for this thesis. This will include a rationale for the study design, the processes involved in data collection and the statistical methods used in data analysis.

3.2 Local Recurrence in Patients with Narrow Surgical Margins

3.2.1 Study Question

The hypothesis of this study is that a narrow (less than or equal to 2mm) surgical resection margin in breast conserving therapy done for stage I and II breast cancer does not result in an increased local recurrence rate.

3.2.2 Study Design

A randomized controlled study to address the issue of narrow surgical resection margins in early stage breast cancer treated with BCT is not possible. To do so one would need to randomize patients to one of two arms—wide excision with a surgical resection margin of greater than two mm and narrow margin excision with a surgical resection margin that was negative but less than or equal to two mm. Even if surgeons were capable of that degree of precision (which they are not) such randomization would be unethical.

It is conceivable that one could design a study where in all women who had a lumpectomy with a surgical resection margin less than or equal to 2 mm. were randomized to further wide excision or no further surgery, controlling for other variables known to be associated with increased local recurrence. This study design would be the one most likely to allow for resolution of the question and to assign causation to the results. Moreover, being prospective, it

would allow for the incorporation of newer molecular methods of tumor classification to be part of the final analysis. While such a randomized prospective study would offer the strengths mentioned above, there are several challenges that would make it very unlikely to be able to successfully conduct such a study. The 10 years it would take to wait for results would itself be a serious challenge to conducting such a prospective study. Furthermore, given that the question being asked is about local recurrence and not survival it is unlikely that the required funding to perform the study could be found. Finally, it would be difficult to recruit enough people to agree to be randomized into the two arms of the study (re-excision versus no further surgery) in the absense of adequate literature on the topic to stress the need for such a randomized study. The perception that one should "do everything" would almost certainly hamper recruitment.

Having accepted that a prospective randomized trial was not feasible the study design felt most appropriate in the hierarchy of study designs was that of a retrospective cohort design. This study design was deemed feasible and was therefore chosen.

3.2.3 Inclusion and Exclusion Criteria

In order to minimize bias in the selection of the two cohorts inclusion and exclusion criteria were established. The patients included in the study were patients with a diagnosis of invasive duct carcinoma of the breast in stage I or II as defined by the American Joint Committee on Cancer seventh edition (AJCC), treated with breast conserving therapy.(225) Breast conserving therapy was defined as surgical removal of the malignant tumor with a negative surgical resection margin and postoperative radiation to the whole breast using a standard radiation protocol. As BCT is standard treatment only in stage I and II breast cancer (early stage breast cancer), inclusion was limited to these patients. The AJCC define tumor stage based on the TNM system where T refers to tumor size, N refers to the nodal status and M stands for the metastatic status. Stage I breast cancers are composed of all T1N0M0 cancers, where T1 refers to tumors 2

cm. or less in greatest diameter, N0 indicates no regional modal metastasis, and M0 indicates no distant metastasis.. Stage II breast cancers are divided into two groups—IIA and IIB. Stage IIA cancers are those which are T0N1M0, T1N1M0, or T2N0M0 while stage IIB cancers are those which are T2N1M0 or T3N0M0. T0 indicates that no tumor is identified in the breast, T1 refers to cancers less than 2 cm in diameter, T2 refers to cancers more than 2 but less than 5 cm in maximum dimension, and T3 refers to cancers more than 5 cm. N1 refers to metastasis in 1 to 3 axillary lymph nodes.

There are several different histological diagnosis of breast carcinoma that imply either a better or worse prognosis.(138) Medullary carcinoma accounts for about 4% of all breast cancers and is characterized histologically by a dense lymphoreticular infiltrate composed predominately of lymphocytes and plasma cells, large pleomorphic nuclei that are poorly differentiated and show active mitosis, and a sheet-like growth pattern with minimal or absent ductal or alveolar differentiation. This histologic type is a frequently seen in BRCA-1 hereditary breast cancer and has a better five year survival than invasive duct carcinoma. Mucinous or colloid carcinoma accounts for about 2% of all breast cancers characterized by extracellular pools of mucin surrounding aggregates of low-grade cancer cells. Papillary carcinoma is another good risk breast cancer that makes up about 2% of the overall total of invasive breast cancer. These tumors are characterized by papillae with fibrovascular stalks and multilayered epithelium. Tubular carcinoma is another special histologic group making up about 2% of the total and carrying a better prognosis. Micropapillary carcinoma is a more recently recognized histologic type which carries with it a poorer prognosis.

Several other rare histologic types have been described. Amongst the carcinomas acinic cell carcinoma, adenoid cystic carcinoma, and squamous cell carcinoma are described. In addition

one can occasionally find a primary lymphoma of the breast or a primary mesenchymal tumor (sarcoma). In recent years the boundaries between histologic classification of tumors and the genetic classification has become blurred with the description of a Basal-like carcinoma. This entity is characterized by a strongly staining basal group of cells and by staining negatively for estrogen receptors, progesterone receptors and the Her2 receptor and frequently corresponds to basal like genetic type. It carries a worse prognosis than other breast cancers.

Lobular carcinomas make up about 10% of all breast cancers and clinically are associated with multifocality, multicentricity and bilaterality. While the prognosis of lobular carcinoma is similar stage for stage with invasive duct carcinoma the association with multifocality and multicentricity would make it difficult to compare this tumor subset with invasive duct carcinoma in terms of local recurrence.

In order to avoid the bias of one group having more patients with a better or worse prognosis tumor inclusion was restricted to the most common histological diagnosis of invasive duct carcinoma.

3.2.4 Sample Size Calculation

The World Health Organization manual entitled "Sample size determination in health studies. A practical manual" was used to aid in determining the sample size required.(226) The formula used was $n = \frac{\left\{Z_{1-\alpha/2}\sqrt{\left[2\bar{P}(1-\bar{P})\right]} + Z_{1-\beta}\sqrt{\left[P_1(1-P_1)+P_2(1-P_2)\right]}\right\}^2}{(P_1-P_2)^2}$ where $\bar{P} = \frac{(P_1+P_2)}{2}$. The test value of Relative Risk (RR) was considered to be 1, where Relative Risk = P_1/P_2. The anticipated probability of recurrence given a wide surgical resection margin was considered to be 10% at 10 years based on the literature (P_1=0.10). The anticipated probability of recurrence given a narrow surgical resection margin was considered to be 15% (P_2=0.15). The anticipated relative

risk was 0.5. The level of significance was chosen to be 5% (p=.05) and the power of the test was fixed at 80% (80% confidence of correctly rejecting the null hypothesis (RR=1) if it is false). For a two-sided test the sample size calculation determined that 200 patients would be required in each arm of the study (narrow margin and wide margin) to detect a 50% difference in LR assuming a baseline 10% incidence of LR over 10 years.

3.2.5 Definition of Local Recurrence

Local recurrence was defined using a modification of that offered by Abd-Alla et al. in which an ipsilateral breast recurrence was considered a true recurrence if it was located within 3 cm of the primary tumor bed and had a histological subtype consistent with the primary tumor.(47) Given the retrospective nature of this study it was impossible to determine if a given recurrence was within 3 cm of the primary tumor bed. Consequently the definition used in this study for a local recurrence was an ipsilateral breast recurrence that had a histological subtype consistent with the primary tumor. Tumor recurrence in the opposite breast, metastatic breast recurrence, nodal recurrence, tumors where the "recurrence" was of a different histology from the primary, chest wall recurrence without recurrence in the breast parenchyma and isolated skin recurrences were not considered to be local recurrence.

3.2.6 Study Period

To adequately examine the question of local recurrence it was decided that a minimum10 year follow up period was required. Local recurrence in early stage breast cancer is a cumulative event with increasing incidence over time. Most studies in the literature that examine the question of local recurrence in breast cancer consider 10 years to be adequate and that was accepted as a reasonable time frame to consider.

All patients treated at the Saskatoon Cancer Agency during the years of 1991-2001 that fit the inclusion criteria were included in this study. This time frame was chosen for three reasons. First,

by ending the data collection in 2001, the chosen timeframe allows for a minimum 10 years of follow-up. The start date established for our data collection was determined by a combination of the above sample size calculation described above, and an estimation of the number of patients that would meet the inclusion criteria that was based on an initial review of the number of cases being registered at our Cancer Center. The sample size calculation combined with the initial review led us to predict a need for 10 years worth of patient data. Finally, during the time period chosen it was not standard practice to re-excise narrow margin specimens; hence an assumption was made that it would be possible to accrue the required number of narrow margin cases.

3.2.7 Radiation Dose

The standard radiation dose given was 5,000 Gy administered to the whole breast over a 5 week period divided into 25 daily sessions of radiotherapy Monday to Friday with a rest on the weekends. The standard boost of radiation was a further 1,000 Gy given over 5 days.

3.2.8 Ethics Approval

Ethics approval was obtained from the University of Saskatchewan Health Research Ethics Board (#08-60) as well as the Saskatchewan Cancer Agency and the Saskatoon Health Region.

3.2.9 Data Collection

The charts of all patients treated at the Saskatoon Cancer Agency during the years of 1991-2001 with a diagnosis of stage I or II breast cancer and a histological diagnosis of invasive duct carcinoma of the breast were reviewed to determine whether the inclusion criteria were met. Patients who went on to have a mastectomy, who had a different diagnosis, who had a more advanced stage, who had a positive resection margin or who did not have radiation treatment were excluded from further analysis. For the remainder the last date of follow-up or local recurrence was recorded as the primary endpoint and was considered the dependent variable in the statistical analysis.

The margin status was determined by reviewing the pathology report. In cases where the pathologist had recorded the margin this was taken to be accurate and valid. This assessment was done on formalin fixed, paraffin embedded specimens. In cases where there was a re-excision and there was no residual tumor in the re-excised specimen the margin was considered to be greater than 2mm and was recorded as " wide margin on the basis of re-excision". In cases where the margin status could not be determined from the chart the slides were retrieved and independently reviewed. In those cases where the pathologic review could not be determined this was recorded as being unable to assess.

Based on the literature survey other variables considered to be associated with LR included estrogen receptor status, tumor grade, tumor size, age, the presence of extensive duct carcinoma in situ, radiation boost, and adjuvant chemo or hormonal therapy. Data on these variables were collected. Other information gathered included the date of surgery (which was used as a surrogate for the date of diagnosis), the name of the surgeon, the name of the pathologist, follow up details including the date of death or last follow-up visit, the presence or absence of metastatic disease (and if so when), and the management of any local recurrence (none, wide excision, mastectomy, further chemo/hormonal therapy), and any new or other primary cancer diagnosis.

The data was collected on individual data sheets and the information transcribed into a database created using Microsoft access.

3.2.10 Statistical Analysis

3.2.10.1 Overview

The dependent variable in this study is dichotomous (local recurrence or no local recurrence). In addition there are several variables that need to be considered as possible confounding or interacting causes of local recurrence in early stage breast cancer treated with BCT. In order to

address the main variable of interest and other possible variables logistic regression analysis was the appropriate statistical analysis to use. Given that recurrence over time was the end point survival analysis both with the Kaplan-Meier technique as well as the Cox Proportional Hazards Model were also conducted. All statistical analysis was conducted with the aid of the SPSS 18 software package.

3.2.10.2 Logistic Regression

Linear regression is not an appropriate statistical methodology to test the strength of an association between two variables when the dependent variable is dichotomous. As in many health science related research this study's variable of primary interest or dependent variable (local recurrence) was dichotomous. Logistic regression is a mathematical modeling approach that can be used to describe the relationship of several X's to a dichotomous dependent variable. While other modeling approaches are possible, logistic regression is by far the most popular because the logistic function, on which the model is based, provides an estimate that must lie in the range between zero and one and it creates an appealing S-shaped description of the combined effect of several risk factors on the risk for a disease. In logistic regression two questions are addressed. The first question is whether or not an association exists between two variable of interest and the second is the strength of that association. Chi-square analysis is used to answer the first question and the odds-ratio to answer the second.(227)

In order to create a linear transformation of the logistic model an alternative way of writing the model is first performed. This form of the model is called the logit form. Denoted as logit *p(x)* this form is produced by the natural log of the quantity *p(x)* divided by one minus *p(x)*, where *p(x)* denotes the logistic model. The mathematical equation is $\log[p(x)/1\text{-}p(x)]=\log(odds)=\beta_0+\beta_1 *x.$ The odds ratio is then derived from this equation wherein $\log[p(x)/1\text{-}p(x)]=\log(odds)=\beta_0+\beta_1 *x.$(227)

In logistic regression the least squares estimators for the model parameters are not used because variance of the dependent variable is not constant across the values of the predictor variable, the least squares estimates are not efficient and the standard error estimates are not consistent. Instead the maximum likelihood method is used to produce estimators that are consistent, asymptotically efficient and asymptotically normal.(227) The likelihood function that was thus created achieves that.

In logistic regression the likelihood ratio test (LRT) is used to compare two models that contain different variables of interest fitted to the same data by the maximum likelihood method. The LRT is used to test the null hypothesis that the model with more parameters is not a significantly better model than the model with fewer parameters. By comparing different models one is able to find the one that best describes the relationship between the dependent variable and a number of independent variables of interest. (227)

In addition a researcher needs to consider the possibility of both confounding and interaction of variables. Confounding exists if meaningfully different interpretations of the relationship of interest occurs when an extraneous variable is ignored or included in the regression mode. An assessment of confounding is done by comparing a crude and an adjusted estimate of association (the crude estimate ignores the extraneous variable of interest while the adjusted estimate includes it). A meaningful difference is, in the end, a clinical judgment but generally a 20% difference between is considered meaningful. Interaction is tested for by adding an interaction term to the model and comparing models using the LRT to see if the addition of the interaction term contributes significantly to the prediction of the outcome variable.(227).

For this study therefore, first a univariate analysis was performed for each variable of interest. For each variable considered the data was analyzed at the 10 year follow-up point. For

categorical variable a chi-square test was conducted whereas a t-test was used to examine continuous variables. Following the univariate analysis all variables that were either clinically important based on the literature review or those with a $p \leq .20$ were included in the multivariable analysis or statistical modeling. Using the Likelihood Ratio Test various models were compared to arrive at the best model that predicted Y (local recurrence). Having completed that further modeling was done to take into account the possibility of confounding and/or interaction effects.

3.2.10.3 Kaplan-Meier Survival Curve

When the outcome variable of interest is time until an event occurs the data can and should ideally be analyzed using a collection of statistical procedures collectively called survival analysis. Censoring (a key data analytical problem) occurs when we have some information about individual survival time but we do not know the survival time exactly. Using the intuitive approach of either using follow-up time regardless whether it is censored or not, or only using complete data results in underestimating the survival time either by reducing survival time or by selection bias. Survival curve estimates can either be achieved by using a life-table estimate that use grouped or ungrouped data and estimates survival at arbitrary intervals or by using a product-limit estimate (the Kaplan-Meier estimate) that uses ungrouped or individual data and estimates survival at each time an event is observed. The Kaplan-Meier method was chosen to analyze the data in the study done for this thesis. While there was a minimum follow-up period of 10 years some patients were followed as long as 20 years. The log-rank test was performed to test for statistical equivalence between the curves generated. This test is a large-sample chi-square test comparing the curves.(228)

3.2.10.4 The Cox Proportional Hazards Model

The Cox proportional hazards model is a popular mathematical model used for analyzing survival data that includes multiple variables. A key reason for the popularity of the Cox model is that, even though the baseline hazard is not specified, reasonably good estimates of regression coefficients, hazard ratios of interest, and adjusted survival curves can be obtained for a wide variety of data situations. The Cox proportional hazards model is preferred to logistic regression modeling when analyzing survival data because it takes into account survival times and censoring.(228)

The two primary quantities desired from the Cox model are an estimate of hazard ratios and an estimate of survival curves. A hazard ratio (HR) is defined as the hazard for one individual divided by the hazard for a different individual. When a Cox model is used to fit survival data, survival curves can be obtained that adjust for the explanatory variables used as predictors, with the value chosen for the adjusted covariate typically being the mean value.

The Cox model makes use of maximum likelihood (ML) estimates generated from a computer program (in this case SPSS 18). As with logistic regression the ML estimates of the Cox model parameters are derived by maximizing a likelihood function, which is a mathematical expression describing the joint probability of obtaining the data actually observed on the subjects in the study as a function of the unknown parameters in the model being considered. The likelihood ratio test can be used in the Cox model very much like the likelihood ratio test for the logistic regression model, where we compared the -2log likelihood values between different models. Variable selection for the various models is the same as is used for logistic regression.

The underlying assumption of the Cox proportional hazards model is that the hazards are proportional over various time points. This implies that the cumulative survival curves and the hazard curves for groups do not cross. This assumption needs to be checked for validity in order

to use the information derived from the model. There are three general approaches that can be used to assess the proportional hazards assumption—graphical, goodness of fit test and using time-dependent covariates.

The dependent variable in this study is dichotomous (local recurrence or no local recurrence). In addition there are several variables that need to be considered as possible confounding or interacting causes of local recurrence in early stage breast cancer treated with BCT. In order to address the main variable of interest and other possible variables logistic regression analysis was the appropriate statistical analysis to use. Given that recurrence over time was the end point survival analysis both with the Kaplan-Meier technique as well as the Cox Proportional Hazards Model were also conducted. All statistical analysis was conducted with the aid of the SPSS 18 software package.

3.2.10.5 Variable coding

The coding of the variables are found in table 3.1. In some of the cases included in the study p53 status and Bcl-2 status was available. Given that this information was available in only a minority of the cases and given that the literature did not support them being significant independent variables they were not included in the analysis. The margin of resection was recorded categorically as four categories. The narrow margin group was divided into less than or equal to 1 mm and 1-2 mm. so that the different narrow margin widths could be interrogated even though it is acknowledged that the two sub-groups need to be taken together to attain adequate power based on the sample size analysis. The wide excision group was divided into two based on how the designation of wide excision was achieved (by initial resection or re-excision). This was done because of some literature suggesting that re-excised tumors might have an increased local and distant failure. While tumor size as a variable was recorded as a continuous variable it was decided to examine this as a dichotomous variable based on T size as has been

done elsewhere in the literature in order to allow for comparison. Likewise age was made into a dichotomous variable as is the norm in the literature. Age 40 was chosen for the cut off point for "young age". All patients 40 years of age or less were considered young while those over the age of 40 were not. Estrogen receptor status was considered positive if 10% or more of the nucleus stained positive. This cut-off reflects the sensitivity of the test available at the time the data was collected.

Table 3.1 Coding of dependent and independent variables.

Variable	Coding	Type of variable
Dependent variable (Y)		
Local recurrence of breast cancer	No=0 Yes=1	dichotomous
Independent variables (X)		
Surgical resection margin (X1)	Less than 1mm=0 1-2 mm=1 greater than 2 mm (measured)=2 greater than 2 mm (re-excised)=3	categorical
Radiation boost (X2)	No=0 Yes=1	dichotomous
Tumor grade (X3)	Grade 1-0 Grade 2=1 Grade 3=2	categorical
Tumor size (X4)	≤ 2 cm =0 2.1-5 cm =1	dichotomous
Extensive DCIS (X5)	No=0 Yes=1	dichotomous
Lymphovascular invasion (X6)	No=0 Yes=1	dichotomous
Estrogen receptor status (X7)	Negative=0 Positive=1	dichotomous
Progesterone receptor status (X8)	Negative=0 Positive=1	dichotomous
Her2 status (X9)	Negative=0 Positive=1	dichotomous
Age (X10)	≤ age 40 =0 >age 40 = 1	dichotomous
Chemotherapy or hormonal therapy (X11)	None =0 Hormonal therapy = 1 FAC =2 Other chemo = 3	categorical
Lymph node status (X12)	None =0 Some =1	dichotomous

CHAPTER 4
RESULTS

4.1 Introduction

In the 10-year study period 3960 patients were registered with the Saskatoon Cancer Agency with a diagnosis of breast cancer. From the cancer center registry of these 3960 patients it could be determined that 2,872 were either stage III or IV, had a mastectomy as their initial surgical procedure, had a diagnosis other that invasive duct carcinoma or failed to receive adjuvant radiation. These 2,872 patients were excluded from further analysis. The charts were reviewed in all of the remaining 1088 patients.

Of the charts reviewed a further 263 were excluded. The majority of these were excluded because they went on to a mastectomy after their initial segmental resection. Other reasons for exclusion included: misclassification (wrong diagnosis, advanced stage, incomplete or no adjuvant radiation) or inability to locate the chart in 25. The details of these exclusions are found in table 4.1. In the remaining 825 charts there were 312 in which the surgical margin was measured in the original pathology report (164 greater than 2 mm and 148 less than or equal to 2 mm), 249 in which there was a re-excision in which no residual tumor was detected, and 264 cases in which the report was unclear and required an independent pathologic review. Amongst the 264 reviewed cases 134 were unable to be assessed either because the slides were lost, the margin was never marked, or the stain had faded to an extent that it was impossible to tell. Figure 4.1 shows the exclusions that were made based on the registration data. Table 4.1 shows the further exclusions that occurred after the charts were reviewed including the reasons for the exclusions. Figure 4.2 takes a closer look at the 1088 patients that were included in the study and the disposition of those cases.

Figure 4.1 Chart depicting patient inclusion/exclusions based on registration data

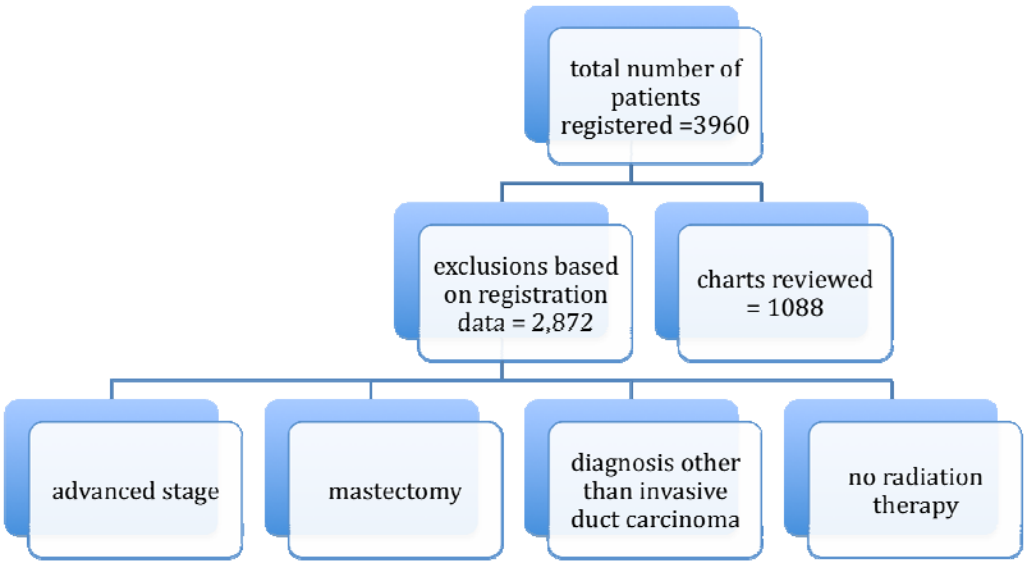
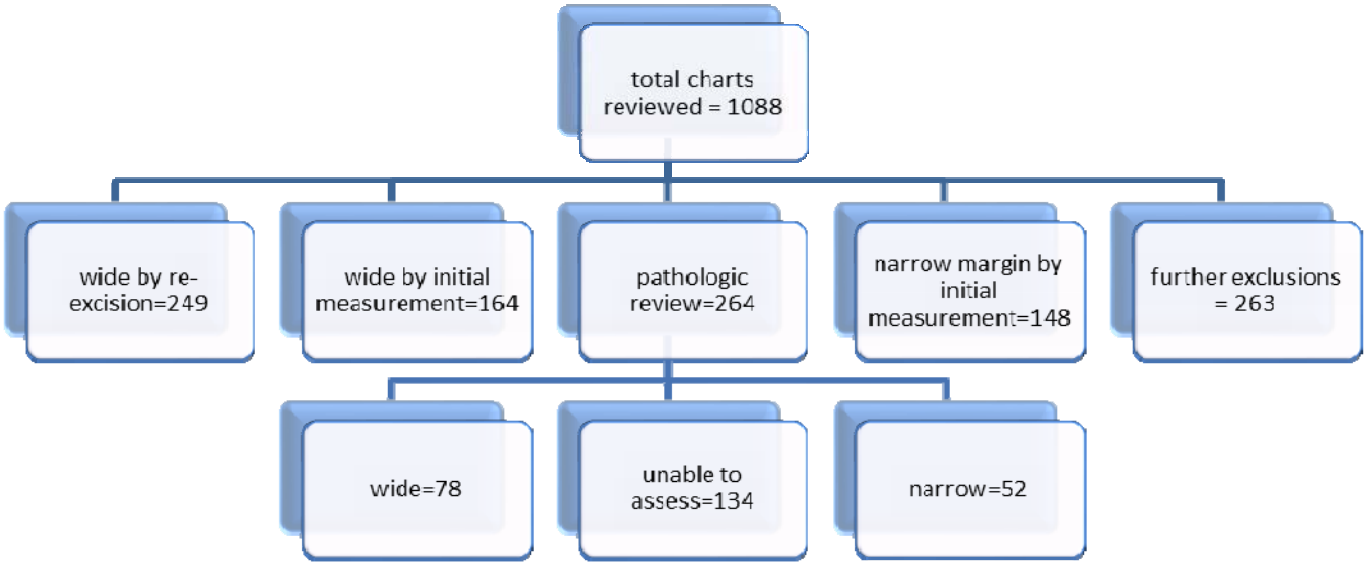


Table 4.1 Reasons for exclusions following chart review

Indication	**Number**
Went on to a modified radical mastectomy	215
Wrong diagnosis	6
No radiation	3
Positive margin	2
Outside of study time frame	2
Surgery out of country	1
Advanced stage	8
Unable to locate the chart	25
Total	263

Figure 4.2 The disposition of the 1088 reviewed charts.



4.2 Data Collection.

In reviewing the charts demographic information as well as detailed information from the pathology report and follow up data was collected. The following was collected for each chart:

identifying data (name, cancer center chart number, hospitalization number)

date of birth

date of diagnosis

histologic diagnosis

tumor size in cm

ER status

PR (progesterone receptor) status

tumor grade

adjuvant radiation treatment

adjuvant chemotherapy or hormonal therapy

recurrence and if so the details of what kind of recurrence and when

date of death or last follow-up

presence or absence of extensive duct carcinoma insitu

margin status

name of the pathologist

name of the surgeon

A database was created using Microsoft access and all of the above data was entered.

The data that was available on age was continuous. The literature generally considers age of 40 as the discriminating point with younger age women having a higher risk of local recurrence. In doing our assessment age was examined both as a dichotomous variable (less than or equal to 40 or greater than 40) as well as a continuous variable. Age was available for all cases.

As mentioned in the literature survey grade was divided into 3 categories according to the modified Bloom-Richardson grading system. Higher grades are associated with increased local recurrence. Grade was available for all cases.

During the study period examined a number of different chemotherapy regimes were used. Some women received Tamoxifen only (an anti estrogen drug), some received Taxotere based chemotherapy and the majority received FAC (5-flurouricil, adriamycin, and cyclophosphamide). The breakdown is seen in Table 4.2. Because of this variation the univariate analysis for adjuvant therapy was done in more than one way. The simplest examination was dichotomous—adjuvant therapy or not. The data was then analyzed broken down into 3

categories with 0=nothing 1=hormonal therapy and 2= chemotherapy. There were no cases missing data. The literature demonstrates that adjuvant chemotherapy and hormonal therapy is associated with less local recurrence. Only 24.6% of our patient population received adjuvant hormonal therapy and another 20.9% received adjuvant chemotherapy. Those numbers are low compared with what one would see in a modern oncology practice.

Table 4.2 Descriptive analysis of adjuvant systemic therapy usage

Type of treatment	**Number of patients (%)**
None	449 (54.4)
Hormonal	203 (24.6)
Chemotherapy--total	173 (20.9)
Chemotherapy--FAC	44 (5.3)
Chemotherapy--CMF	87 (10.5)
Chemotherapy--other	42 (5.1)

Both positive estrogen receptor status and progesterone receptor status have been associated with better prognosis and decreased local recurrence. The receptor status was available for the majority of cases examined and where it was missing this was recorded as missing using 99. Both of these were examined as dichotomous variables with 0=negative and 1=positive. In the majority of cases the estrogen and progesterone status was recorded as either positive or negative. In a minority of cases an immunohistochemisty score of 0-4 was recorded in which case any score of 1-4 was considered positive. During the time period that data was collected for this study a positive score equate to 10% or more of the cells being positive for the receptor.

Radiation boost to the tumor bed was not a common practice during the study period. It is now recognized that a boost of radiation to the tumor bed can decrease local recurrence from about 10% in 10 years to 6% in the same time period but at a cost in terms of side effects. Radiation boost was examined as a dichotomous variable with 0=none and 1=radiation boost.

The presence or absence of extensive duct carcinoma in situ (DCIS) was available. An assumption was made that where it was not recorded as such it did not exist but this assumption may or may not be a valid as extensive DCIS was not routinely reported prior to 1998 in Saskatoon. Extensive DCIS is associated with increased local recurrence. This was examined as a dichotomous variable with 0=none and 1=positive. DCIS at the margin was not routinely reported.

It was a bit challenging to decide how best to measure margin status, the primary independent variable of interest. Ideally an independent expert breast pathologist would have reviewed all of the pathology by pulling the old slides and re-examining them. Unfortunately this was not practical with the number of cases that we had. In consultation with our breast pathology expert it was decided that cases where the original pathologist measured the margin or stated that the margin was "widely clear" we would assume that the report was an accurate reflection on reality. In all cases where there was a re-excision and there was no further residual tumor identified in the re-excised specimen it was decided that this likely represented a wide surgical resection margin, but to reflect the assumptions made these cases were recorded as a wide excision by virtue of it being re-excised. All other cases had the original sides pulled and reviewed by one of two pathology residents supervised by Dr. H. Reese, our local breast pathology expert. In this later group of patients some of the slides could not be located, in some cases the ink had faded so badly that the margins could not be assessed and in other cases the margin was never inked

making margin assessment impossible. These cases were classified as “unable to assess” and excluded from further analysis. The breakdown of the re-examined cases is found in Table 4.3.

Table 4.3 Overview of the 264 pathologically reviewed slides

Unable to assess	Wide margin	Narrow margin
34	78	52

While data was collected on p53 status, Bcl-2 status, and Her2 status the vast majority of cancers did not have these markers tested and as such it was not feasible to include them in the multivariable analysis. None of these were associated with increased or decreased local recurrence in the existing literature and therefore this was considered a reasonable decision to make.

Table 4.4 lists the various margin categories broken down into local recurrence or not along with the percentage of local recurrence for each category.

Table 4.4. Local recurrence rates for narrow and various wide excision groups

	Local recurrence (%)	Total	% Local recurrence
Narrow margin	19	201	9.5%
< 1 mm	5	47	10.6%
1-2 mm	14	154	9.1%
Wide margin	56	500	11.2%
measured	21	261	8 %
by re-excision	35	239	14.6%
Total	75	701	

4.3 Data Analysis

4.3.1 Overview

Data analysis was done using logistic regression, Kaplan-Meier survival curve and the Cox Proportional Hazards Model.

4.3.2 Logistic Regression

Logistic regression was chosen because the dependent variable, local recurrence, was dichotomous. That is to say, either there was local recurrence or not (0=no local recurrence and 1= local recurrence).

First the independent variable of primary interest (margin status) was examined in relation to the dependent variable (local recurrence) at 10 years. Narrow margin status was considered as less than or equal to 2 mm. This groups was further divided into less than or equal to 1 mm and 1-2 mm but given the small numbers in each of these two groups (Table 4.4) the analysis comparing narrow margins was done with the two groups combined in order to have adequate numbers to meet the sample size calculation done earlier. Because of concerns about the validity of assuming that a wider excision without residual cancer in the re-excised specimen could safely be considered a true wide excision it was decided to do this analysis in two ways. In the first analysis the measured and re-excised wide margins were grouped together into one group called wide margin. In this scenario the two dichotomous groups were 0=less than or equal to 2 mm resection margin and 1 = greater than two mm resection margin. This showed that margin of resection did not significantly contribute to the prediction of local recurrence (p=.499). In a second run 3 categories of assessment for this dichotomous independent variable (0=less than or equal to 2 mm resection margin, 1=greater than 2 mm measured by a pathologist, 2=greater than 2 mm by virtue of a wider excision in which there was no residual tumor). The univariate analysis of this independent variable of primary interest is shown in Tables 4.5, 4.6 and 4.7. This

univariate analysis showed that re-excised wide margins compared with measured wide margins did significantly contribute to the prediction of local recurrence (p=.02) while narrow margin compared with either measured or re-excised wide margins did not.

Table 4.5 Statistical analysis of local recurrence by margin status --wide excision vs. narrow margin

Margin	OR (CI)	p value
Narrow margin vs wide margin measured and re-excised	1.208 (.698-2.090)	0.499

Table 4.6 Statistical analysis of local recurrence by margin status with narrow margin as the reference group compared with wide excision measured and re-excised wide excision

Margin	OR (CI)	p value
Narrow margin vs measured wide	0.838 (.48-1.605)	0.594
Narrow margin vs re-excised wide	1.643 (.908-2.974)	0.101

Table 4.7 Statistical analysis of local recurrence by margin status with re-excised wide margin as the reference group compared with narrow margin and measured wide margin

Margin	OR (CI)	p value
Re-excised wide margin vs. narrow margin	0.608 (.336-1.101)	0.101
Re-excised wide margin vs. measured wide margin	0.510 (.288-.904)	0.021

The second step taken was to do a univariate analysis of the various independent variables that had been identified in the literature as having an association with local recurrence (age < 40, grade, adjuvant chemo or hormonal therapy, estrogen and progesterone receptor status, radiation boost, tumor size, and the presence of extensive duct carcinoma in situ). This univariate analysis is presented in Table 4.7. Only re-excised wide margin compared to measured wide margins,

high grade compared with low grade and age <40 were found to be statistically significant in that univariate analysis.

Table 4.8 Univariate analysis of variables

Variable	Odds ratio (CI)	p value
size (T1 vs T2)	1.401 (.844-2.325)	0.139
Size in cm with <1 as reference		
< 1cm	1	
1-2 cm	.995 (.599-1.653)	0.948
2.001-3 cm	1.16 (.589-2.285)	0.667
3.001-4 cm	2.011 (.811-4.988)	0.131
4.001-5 cm	2.463 (.488-12.419)	0.275
Size in cm with 4.001-5 cm as reference		
< 1cm	.406 (.081-2.047)	0.275
1-2 cm	.404 (.081-2.012)	0.268
2.001-3 cm	.471 (.089-2.497)	0.376
3.001-4 cm	.817 (.139-4.813)	0.823
4.001-5 cm	1	
Grade with high grade (grade 3) as reference		
grade 1	.406 (.214-.770)	0.006
grade 2	.679 (.393-1.171)	0.164
grade 3	1	
Grade with low grade (grade 1) as reference		
grade 1	1	
grade 2	1.673 (.929-3.012)	0.086
grade 3	2.466 (1.299-4.680)	0.006
Margin of resection with re-excised wide margin as reference		
narrow	.608 (.336-1.101)	0.101
wide	.510 (.288-.904)	0.021
wide by re-excision	1	
Margin of resection with narrow margin as reference		

Variable	Odds ratio (CI)	p value
narrow	1	
wide	.838 (.438-1.605)	0.594
wide by re-excision	1.643 (.908-2.974)	0.101
Extensive DCIS (yes/no)	.596 (.291-1.220)	0.157
DCIS status with extensive as reference		
none	.648 (.314-1.336)	0.24
focal	.393 (.157-.981)	0.045
extensive	1	
DCIS status with none as reference		
none	1	
focal	.606 (.313-1.175)	0.138
extensive	1.543 (.749-3.180)	0.24
Nodal status (positive vs. negative	1.458 (.908-2.342)	0.119
ER status (positive vs. negative)	1.003 (.991-1.016)	0.595
PR status (positive vs. negative)	1.002 (.989-1.014)	0.805
Radiation boost (yes vs. no)	1.360 (.702-2.634)	0.362
Chemotherapy/hormonal therapy status (yes vs. no)	1.087 (.701-1.686)	0.708
Chemotherapy groups with other chemotherapy as reference		
hormonal	.388 (.155-.971)	0.043
AC	.671 (.211-2.130)	0.499
CMF	.490 (.174-1.379)	0.177
other	1	
Chemotherapy groups with hormonal as reference		
hormonal	1	
AC	1.728 (.639-4.668)	0.281
CMF	1.262 (.540-2.954)	0.59
other	2.574 (1.030-6.437)	0.043
Age (less than or equal to 40 vs. over 40)	2.821 (1.371-5.804)	0.005

Having completed the univariate analysis all variables with a p value greater than 0.2 or having been identified in the literature as being associated with local recurrence were included in the full multiple variable mode. This full model, which is presented in Table 4.9, included margin status (3 categories with narrow margin as reference), size status (T1 vs T2), age status (less than or equal to 40 vs greater than 40), grade status (3 categories with high grade as reference), chemotherapy/hormonal therapy status (yes vs. no), extensive DCIS status (yes vs. no), nodal status (positive vs. negative) and radiation boost (yes vs. no). The -2LL for the full model was 418.251. The -2LL with no variables was 438.049 with the difference being 19.798. The tabulated chi square with 10 degrees of freedom is 18.307. Given that the calculated value is greater than the tabulated value we can reject the null hypothesis and conclude that the overall model does predict local recurrence. From this point further models in which a variable was removed to identify which variable(s) contributed most to predicting local recurrence were constructed. This data is presented in Table 4.10.

Of all the variables only high grade compared with low grade significantly contributed to the overall model. The remainder of the variables could be eliminated with no effect. Therefore the most parsimonious model is the one that includes resection margin (with narrow margin as reference as it is the research variable of interest) and grade (with high grade as reference). Given that re-excised wide margin compared to measured wide margin appeared to be significant in the univariate analysis a model using re-excised margin as reference was also constructed and compared with the full model using narrow margins as a reference. This did not show the re-excised wide margin to significantly add to the full model. Each variable was subsequently fit into a model testing for interaction between it and the main variable of interest (margin status) and no significant interaction effects were identified.

Table 4.9 Details of the full model of the multivariable logistic regression analysis for local recurrence in early stage breast cancer

Variable	Multivariable OR (CI)	p value
Narrow margin vs. wide margin measured	.689 (.334-1.423)	0.314
Narrow margin vs. wide margin re-excised	1.576 (.815-3.044)	0.176
Age (less than or equal to 40)	1.736(.672-4.355)	0.24
Grade 3 vs grade 1	2.832 (1.298-6.178)	**0.009**
Grade 2 vs grade 3	.607 (.323-1.143)	0.122
Extensive DCIS	1.565 (.608-4.027)	0.353
Positive nodal status	1.393 (.790-2.457)	0.252
Adjuvant chemotherapy or hormonal therapy given	1.478 (.814-2.683)	0.199
T2 vs T1 tumor size	1.28 (.681-2.406)	0.443
Radiation boost given	1.407 (.640-3.093)	0.395

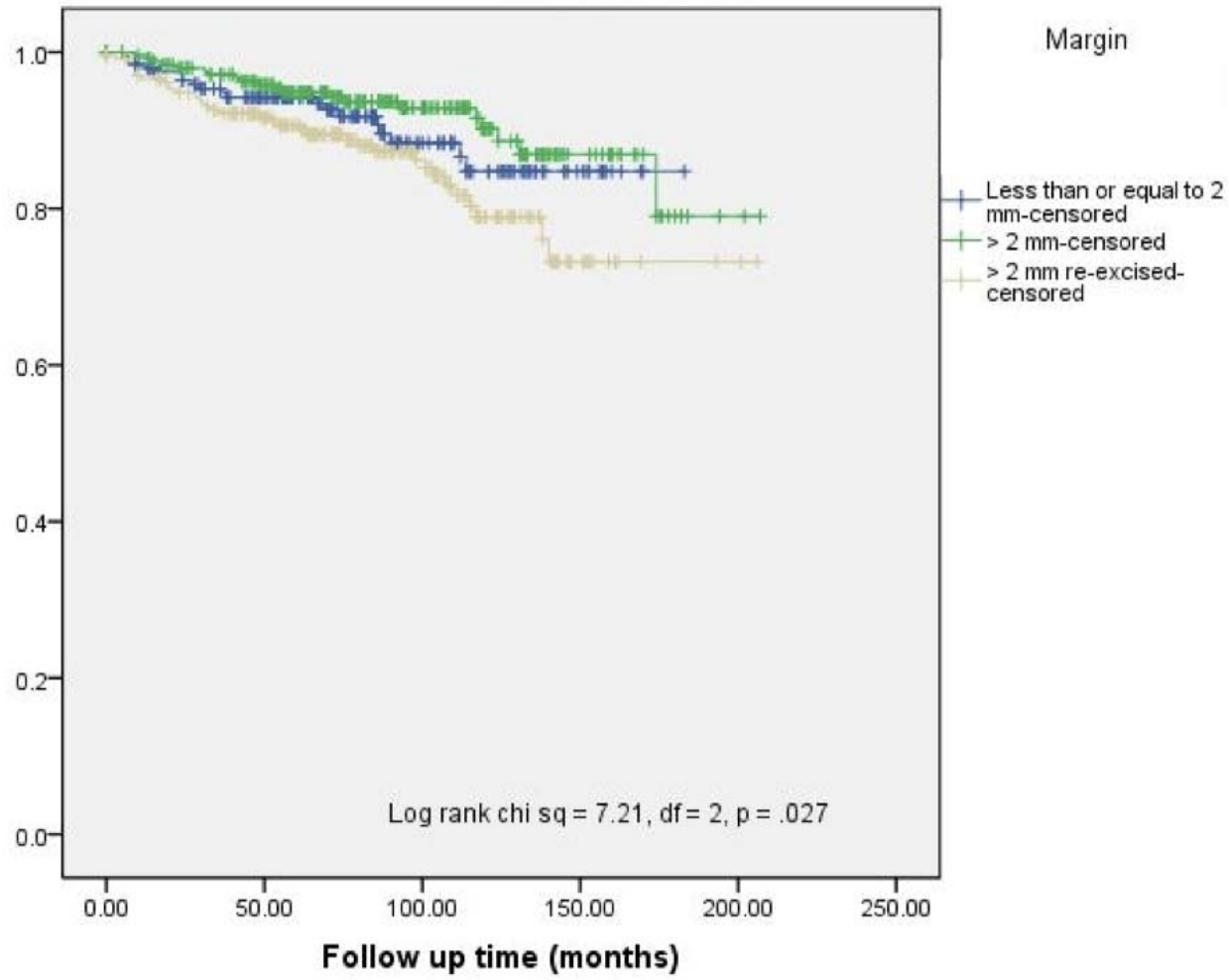
Table 4.10 Results of model building, comparison of the full model to a model with the variable in question being removed.

Variable	-2LL	Full model -2LL	LL	Significant
Size	419.620	418.251	1.369	no
Age	418.313	418.251	.062	no
High grade	463.766	418.251	45.515	yes
Chemo/hormonal therapy	420.040	418.251	1.789	no
Extensive DCIS	418.865	418.251	.614	no
Nodal status	418.960	418.251	.709	no
Radiation boost	418.765	418.251	.514	no

4.3.3 Kaplan-Meier Survival Curve

A Kaplan-Meier survival curve was calculated and is presented in Figure 4.3. This shows no statistically significant difference between the narrow and wide margin groups for local recurrence. The follow-up period extended out as long as 20 months.

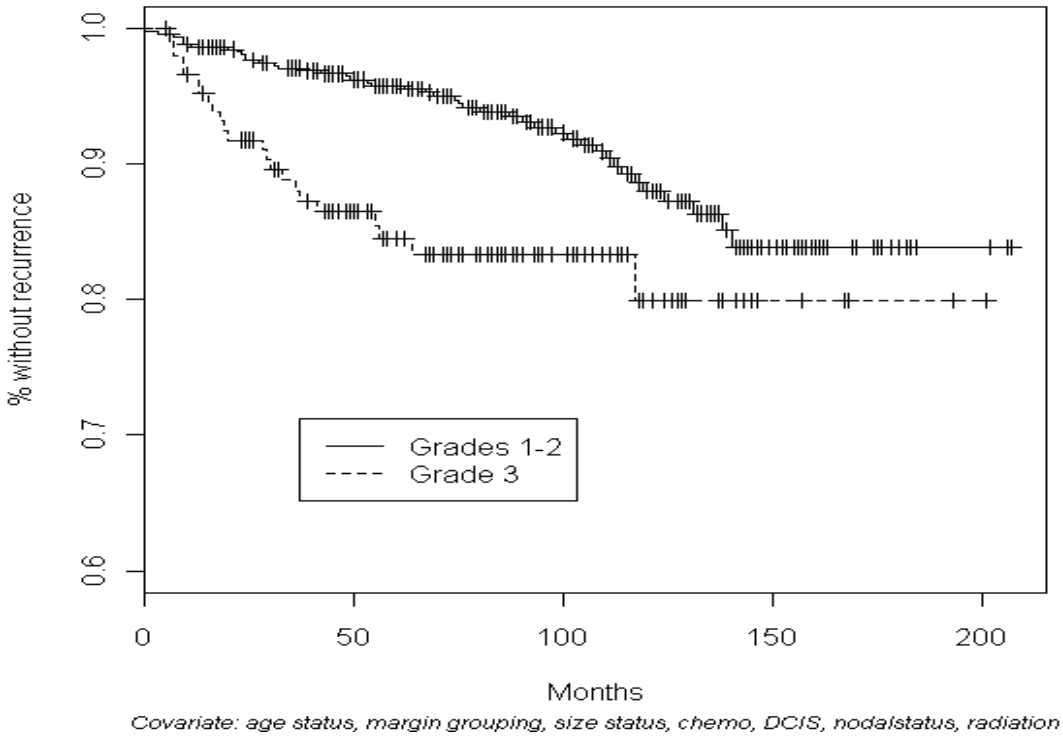
Figure 4.3 Kaplan-Meier Curve Demonstrating Length of Time to Recurrence



4.3.4 Cox Proportional Hazards Model

A Cox proportional hazards model was developed using the variables established for the full model of the logistic regression analysis. Similar model building techniques as were used in the multi-variable analysis were applied to the Cox proportional hazards model analysis. The proportional hazards assumption was met as can be seen in figure 4.4. Margin status (narrow versus wide) did not significantly predict local recurrence. Grade 3 tumors versus either grade 1 or 2 is the only variable that significantly predicted for local control with a hazards ratio of 2.3360 and a confidence interval of 1.3649-3.998.

Figure 4.4 Cox Proportional Hazards Model showing grade alone predicting local recurrence



The results presented in this chapter, other than the Cox proportionate hazards calculations, are also found in the peer reviewed publication of this work in the Journal of Surgical Oncology (Appendix A).

CHAPTER 5
DISCUSSION

5.1 Introduction

The NSABP-06 trial, which ushered in a new era of more conservative surgical management of early stage breast cancer, was published in 1985.(229) Twenty five years later BCT remains one of two primary surgical options for the management of these patients. The problem of local recurrence that was identified early on however continues to vex the medical community. The significance of a local recurrence in the ipsilateral breast increasingly seems to not only be the psychological distress women who have a local recurrence experience and the trade off made in the decision making between BCS and a modified radical mastectomy but also a potential for decreased survival for those women who have a local recurrence. Therefore finding ways to minimize local recurrences in BCT is important. This can be realized in one of three ways. One can improve the treatment techniques in order to decrease the incidence of local breast recurrence, improve the selection of patients who are a candidate for BCS or find ways to better match treatment with the patient—the holy grail of personalized care that we are hearing more and more about in the literature.

As one begins to unwrap these three potential options the complexity of the problem quickly becomes apparent. Early stage breast cancer patients treated with BCS typically have three treatment modalities applied to the management of their disease, both for local control and for the treatment of potential systemic failure and death. While the later has not been the focus of this thesis it is clearly of paramount importance not only as the primary objective of treatment but also in terms of how it interacts with local control.

Radiation therapy has definitively been shown to decrease local recurrence in BCT.(21, 37, 38, 40-42, 70, 71) In order to treat both the current malignancy and to decrease the occurrence of new primary cancers in the ipsilateral breast whole breast irradiation is the standard of care. A boost of 16 Gy of radiation to the tumor bed confers a further reduction in local recurrence rates but at a cost of increased fibrosis and an accompanying decrease in cosmesis.(72)

Systemic treatment with either adjuvant chemotherapy or hormonal therapy was offered to node positive women early on following the discovery of chemotherapy as a treatment option. The early research demonstrated a statistically significant improvement in survival for these women. Over the last quarter century the benefits of systemic adjuvant therapy for at least some node negative women has been explored and shown to be effective.(230, 231) While it is increasingly clear that some node negative women do benefit from systemic adjuvant therapy the current challenge for systemic treatment is to be able to identify those node negative women who will not derive benefit from systemic adjuvant treatment so as to spare those women the cost of going through unnecessary treatment.

Optimizing surgical management is likewise complex. The question of how much surgery is optimal in BCS has been the focus of this thesis. The study conducted for this thesis examining the role of a narrow (less than or equal to 2mm) surgical resection margin in local recurrence significantly adds to the existing literature in providing information to guide the clinical question of whether or not to re-excise lumpectomies with a narrow but negative surgical resection margin. A detailed examination of this will occur in the first section.

It is somewhat surprising that it took 15 years for the literature to recall that a small positive effect can be missed in studies that are underpowered with short follow up periods. Stotter et al.'s article underscored the need for a large randomized cohort of patients (a minimum of

10,0000) comparing BCT with modified radical mastectomy and followed for at least 10 years to allow for the survival equivalence claimed by the NSABP-06 and other similar trials to be valid.(171) It wasn't until the Early Breast Cancer Trialist Collaborative Group published a meta-analysis of randomized trials in 2005 that the survival equivalence of BCT and modified radical mastectomy was examined more carefully.(70) With the results of that met-analysis in hand the problem of local recurrence potentially takes on new significance. The question of whether every woman with an early stage breast cancer should be an equal candidate for BCS or not is no longer just a question of cosmesis and psychological distress but potentially survival as well.

Twenty five years of research examining factors associated with local recurrence in BCS has provided much illumination. We know for instance that women of young age, high tumor grade, lymphovascular invasion etc. are at increased risk of local recurrence. We do not, for the most part, have adequate focus of that illumination however to make use of the information to direct patient care. Ideally we would have markers of one sort or another that individually, or as a set, would allow for the selection of the small cohort of women who will have a local recurrence if submitted to BCS so that they might be directed appropriately to a mastectomy in the first instance. Chapter 7 will explore further avenues of research that might help in this regard.

In attempting to realize the goal of personalized care in breast cancer one must acknowledge just how far we really are in achieving that goal. The first of the "targeted" therapies for cancer was directed at the epidermal growth factor receptor (EGFR). Monoclonal antibodies directed at that receptor have been developed and brought into clinical practice.(232) Imatinib is a derivative of 2-phenylaminopyrimidine that selectively inhibits the tyrosine kinase receptor. This drug was initially developed for the specific inhibition of the fusion gene product BCR-ABL

found in chronic myelogenus leukemia.(233) In 2001 Imatinib was used for a patient with a gastrointestinal stromal tumor with a significant response.(234) Subsequent success in discovering the Her2 receptor and it's targeted therapy Trastuzumab in breast cancer and the development of targeted therapies to vascular endothelial growth factor (VEGF), mesenchymal-epithelial transition factor (C-MET), and Src, insulin-like growth factor-1 receptor (IGF-1R), has fuelled the initial enthusiasm and excitement brought about by molecular targeted therapy.

The reality is that these targeted therapies are applicable to a very small subset of cancers. With few exceptions these therapies as a group have limited application outside of the metastatic palliative arena. It is important—imperative actually—to step back from the busyness of answering highly focused research questions emanating from urgent and pressing clinical problems to ask if we are asking the right questions in the first place. In returning to theory and examining the assumptions that are brought to the table it is hoped that new ideas and research questions can be found.

5.2 Surgical Resection Margin as a Variable to Control in Limiting Local Recurrence in Early Stage Breast Cancer Treated with BCT.

5.2.1 Overview

The study done for this thesis shows that a narrow but negative surgical resection margin in stage I and II invasive duct carcinoma treated with BCT does not result in a greater likelihood of local recurrence compared with wider resection margins (either wider margins by measurement combined with re-excised wider margins, wider margin by measurement alone, or wider margin by re-excision alone). It would follow that re-excision of narrow margin segmental resections is therefore unwarranted.

That would seem to be a bold statement to make following a single retrospective study. To assess the validity of that statement it is important to take a closer look at the whole issue of causal inference in research studies. Much has been written about causality and inference in research, an issue that continues to be revisited intermittently and is an ongoing issue that the health science research community needs to be aware of.

5.2.2 Definition of Causal Inference

Kenneth J. Rothman uses a light switch as an example of how we think about causation in general.(235) When a light switch is turned to the on position a light comes on and when it is turned to the off position that same light turns off. After seeing that association replay itself time and again over many days and weeks it is easy to draw the conclusion that the causal mechanism for getting a light to shine is the light switch. Of course the complete causal mechanism is much more intricate and the switch is only one component of several. Because the switch is the only part of the whole mechanism that needs to be activated to obtain the effect of turning on the light we tend to focus on it as the cause, ignoring the wiring for example. Only when the power goes out in a storm or the light bulb burns out do we consider the other factors (at least some of them).

Parascandola and Weed explore the concept of causation in epidemiology and offer several useful insights.(236) An indepth review of the literature revealed five different ways that cause is defined.

The first of these definitions they call production definitions. Under this definition a cause is something that creates or produces an effect. They point out that this definition is vague, leaving the concepts of production and creation undefined and as a result suggest that a more robust definition is required.

Necessary and sufficient cause definitions are commonly presented in epidemiology. Classical epidemiologic teaching suggests that there are four types of causal relationships,

necessary and sufficient, necessary and not sufficient, sufficient but not necessary and neither sufficient nor necessary. A factor is both necessary and sufficient when the disease in question never develops without that factor and in the presence of that factor the disease always develops. In practice this almost never occurs. Some genetic anomalies such as Down's syndrome would be an excellent example. A factor is consider to be necessary but not sufficient when it is required for a disease to occur but by itself is not sufficient to cause the disease. Infectious diseases are a good example of this as the infection cannot occur without the bacteria in question but not everyone exposed to said bacteria develops the illness in question. With sufficient but not necessary factors there are more than one factor that can cause a disease independently of one another. The criterion of sufficient is difficult to fill even for one factor let alone more working totally independently of each other. It is unlikely that this type of causal relationship is encountered very often. The neither sufficient nor necessary model probably reflects reality the most. This complex model envisions a complex interplay between factors that ultimately cause a particular disease. Moreover it envisions more than one possible combination of factors that might explain the cause of a given disease.

Sufficient component cause definition of causation was articulated by Rothman. Under this perspective cause is made up of a number of components, no one of which is sufficient for the disease on its own. When all of the components are present however a sufficient cause is formed. They take issue with this definition as well arguing that "the sufficient-component cause definition requires that we assume the existence of countless hidden effect modifiers to turn every less than perfect correlation into pure determinism".

A probabilistic or statistical definition of causation forms the fourth type of definition. Under this definition cause is defined as the probability of a disease occurring in an individual exposed

to a given factor. They felt that this type of definition offered the greatest range of possible effects and allows for the possibility that other undiscovered causes may also be at work. They express concern that probabilistic definitions remain unclear about what it means for a factor to increase the likelihood of a disease. Cox, Holland and Olsen take exception to his kind of definition arguing that it is not possible to draw a distinction between causal relations and non-causal associations. Parascandola and Weed make the counter argument that adding a counterfactual element to the definition meets this concern.

A counterfactual statement draws a contrast between one outcome given certain conditions and another outcome given alternative conditions and some statisticians and epidemiologists advocate a definition base on this concept. Counterfactual statements can be either deterministic or probabilistic. Parascandola and Weed argue that "counterfactuals articulate an additional attribute that we suspect will enhance any definition of causation by strengthening the distinction between causation and mere correlation" but go on to dismiss it as a sufficient definition in its own right.

In summary Parascandola and Weed argue for a probabilistic definition of causation combined with a counterfactual condition as the most useful definition.

5.2.3 Study Design and Causal Inference

5.2.3.1 Overview

Experimental designs have traditionally been classified as true experimental, quasi-experimental, pre-experimental and nonexperimental with the true experimental design being the criterion by which all other metholologies are judged.(237) It is this experimental design that is used most often to reveal causal relationships and is held up with the highest regard in determining evidence based practice and developing clinical practice guidelines. True experimental designs are characterized by the prospective randomized study in which subjects

are randomly assigned to either an experimental or control group. Three elements of a true experimental design are random assignment, a control group and manipulation. In health research experimental designs are not always possible, appropriate or ethical in which case quasi-experimental designs are the next best option. In these study designs randomization is absent. Because of the lack of random assignment one needs to more careful in making causal claims, assuring that alternative explanations are considered and the limitations of the study design acknowledged. In pre-experimental designs, in which two of the three criteria of true experimental design are absent, and nonexperimental designs which rely on statistical manipulation of data rather than mechanical manipulation and sequencing causal inference should be avoided.

5.2.3.2 Deriving inferences from epidemiologic studies

The first thing that one does in a study is establish whether or not there is an association between a factor and a disease.(238) Does treatment A work or not, does this exposure result in disease or not, or in the case of this thesis work does a narrow surgical resection margin in BCT for early stage breast cancer result in an increased incidence of local recurrence or not. Relative risk and the odds ratio are two measures of association that are used. Relative risk is the ratio of the risk of disease in exposed individuals to the risk of disease in nonexposed individuals. In case-control studies the only measure of association is the odds ratio whereas in cohort studies both relative risk and odds ratio can be valid measures of association. In cohort studies the odds ratio is defined as the ratio of the odds of development of disease in exposed persons to the odds of development of disease in nonexposed persons. In case control studies the odds ratio the ratio of the odds that the cases were exposed to the odds that the controls were exposed.

Once an association is identified between a factor and an outcome the next question is whether or not that association is a result of a causal relationship. The failure to be clear about

the difference between association and causality costs the collective research endeavor significantly. Gary Taubes explores this problem in an article entitled “epidemiology faces its limits”.(239) From his article it is evident that the chiasm between association and causation is exacerbated by the media (which he describes as the unholy alliance) but a significant onus must be placed on researchers to understand the limitations of the studies they perform. In his article Taubes offers some practical suggestions about when to take a study seriously. He suggests that a combination of a very strong association between a disease and risk factor along with a highly plausible biological mechanism should be present. He goes on to suggest that no single epidemiologic study is persuasive by itself unless the lower limit of its 95% confidence level falls above a three or even four fold risk increase. Consistency is another factor to be considered but is only valuable if the studies use different architectures, methodologies and subject groups and still comes up with the same results.

Hill articulated a set of criteria to aid in making causal inference that are still in frequent use today.(240) These criteria are strength of the association, consistency, specificity, temporality, biological gradient, plausibility, coherence, experimental evidence and analogy. Hill himself and others since have some degree of ambivalence about using a set of criteria like this to pass judgement on when an observed association can be assigned a verdict of causation because its checklist nature risks clouding the logical deductive thought process required to make that assessment.

5.2.3.3 Levels of evidence

Evidence based medicine is by now a commonly articulated concept. Because clinicians need to be able to translate research to the bedside they have had to develop some sort of framework under which they can make recommendations in the common situations where research evidence falls short of being conclusive. The University of Oxford center for evidence based medicine has

a table that provides the reader easy access to the framework and how it works (Appendix B).(241) It incorporates the principles espoused by Hill in 1965 and attempts to fit them into a framework that can be used on a clinical level.

5.2.4 Review of existing literature.

The existing literature addressing the subject of local recurrence in stage I and II breast cancer treated with BCT was grouped according to the type of experimental design. This is presented in Table 5.1 Four of the articles reviewed were not included. Two of these (Freedman et al. and Fredrikson et al.) were excluded because it was unclear what a narrow margin was, one (Chism et al.) because there was no comparison to a wider resection margin group, and the fourth (Vordemark et al.) because their definition of a narrow margin (4 mm.) was so widely divergent from the rest of the literature. With the exception of one study these are all non-experimental studies in which a data set from a cohort of patients underwent statistical interrogation, usually with a logistic regression model or multivariate analysis looking to see if there were any variables that were predictive of local recurrence. There was only one case-control study in this group, the one conducted by Hardy et al.

Table 5.1 Study design in narrow margin studies

Study	Experimental	Quasi-experimental	Non-experimental
Schnitt et al.			Yes
Gage et al.			Yes
Touboul et al.			Yes
Freedman et al.			Yes
Pederson et al.			Yes
Park et al.			Yes
Perez et al. 2003/2010			Yes
Fredrikson et al.			Yes
Smitt et al. 1995/2003			Yes
Chism et al.			Yes
Kunos et al.			Yes
Bollet et al.			Yes
Hardy et al.		Yes	
Jones et al.			Yes
Perez et al.			Yes
Vordermark et al.			Yes
Goldstein et al.			Yes
Karasawa et al.			Yes
Wazer et al.			Yes
Pittinger et al.			Yes
Solin et al.			Yes

Table 5.2 provides an overview of these studies including the number of patients in the study, the number of narrow margin patients and the association between local recurrence and narrow

margins found by the study. Of note the two publications by Smitt et al. and Parez et al. are on the same data set but at different dates.

Table 5.2 Narrow vs. wide margin study details

Study	Number of patients	Number of narrow margin patients	Association with local recurrence
Schnitt et al.	181	25	No
Gage et al.	343	54	No
Touboul et al.	528	21	No
Pederson et al.	1.021	96	No
Park et al.	533	94	No
Perez et al. 2003	1037	56	No
Smitt et al. 2003	535	55	Yes
Kunos et al.	341	22	Yes
Bollet et al.	209	42	No
Hardy et al.	200	52	No
Jones et al.	1616	306	No
Perez et al. 2010	1521		No
Goldstein et al.	607	231	No
Karasawa et al.	941	326	No
Wazer et al.	509	99	No
Smitt et al. 1995	303	17	Yes
Pittinger et al.	211	35	No
Solin et al.	697	35	No

It is clear from the literature compiled to date that the association between local recurrence and narrow surgical resection margins in early stage breast cancer treated with BCT cannot be considered causal. Almost without exception the quality of the study design was non

experimental and as such does not allow for causal inference. Only two studies showed an association between narrow surgical resection margins and local recurrence (one published twice at different time periods). The one case control study available showed no statistical difference between narrow margin groups and wider surgical resection margins. It is disturbing therefore to see authors making statements such as, "it is well established that the extent of surgical resection at the time of BCS influences the risk of local-regional breast cancer recurrence" or "although much debated, 2 mm tumor-free margins have been associated with fewer local-regional breast cancer recurrences after BCS".(152) While these statements are technically true in the sense that positive surgical resection margins (particularly grossly positive margins) are strongly associated with local recurrence and there have been two publications that have found an association between a surgical resection margin of less than 2 mm and local recurrence these statements were made in a manner and context that ignore alternate studies and strongly imply a causative interference.

5.2.5 Current Study

5.2.5.1 Study design

This study was a retrospective cohort study. As mentioned in the methodology section of the study it was considered impossible to conduct a randomized prospective study for this particular research question (one could not randomly assign women to a narrow or wide resection margin—apart from the obvious technical difficulty surgeons would encounter in assuring the person in front of them received the required margin that study design would not be ethical). Conceptually one could prospectively follow all stage I and II patients who had BCS and divide them according to margin of resection and then follow them for evidence of local recurrence for 10 years. Those with narrow surgical resections could even be randomly assigned to re-excision or not. Because of time constraints inherent with such a prospective study and difficulty

anticipated in randomizing the narrow margin lumpectomy patients given the current practice guideline recommendations it was decided to use the next best study design—a retrospective cohort design. This study design, which is quasi-experimental, can allow for causal inference although one needs to be more careful assuring that alternative explanations are explored and limitations acknowledged.

5.2.5.2 Power and sample size

One of the concerns that has not yet been discussed is that of study size and power. Sample size calculations are used in both experimental study designs and quasi-experimental study designs.(237) There are four possibilities to consider when interpreting a study finding. There is no association and the study correctly identifies that, there is no association but the study incorrectly identifies one, there is an association and the study correctly identifies that association or there is an association but the study fails to identify it. Power has been defined as the probability of detecting a difference between two treatments (or in this case narrow and wide margins) if the treatments do in fact differ.(238) The number of patients that need to be enrolled to assure that a negative association is real (and avoid a Type II error) can be calculated. This requires knowledge of the difference in response rates to be detected (a variable which should be clinically significant), an estimate of the response rate in one of the groups, a level of statistical significance or alpha (often .05), the value of the power desired or beta (frequently 0.8) and whether the test should be one or two sided. In this study the sample size calculation for a two sided test, alpha of .05, beta of 0.2 an anticipated difference in local control of 50% and a local recurrence rate in wide excisions of 10% in 10 years revealed that each arm of the study (narrow vs. wide resection) would require 200 patients. That was achieved making this the only study in the literature that is adequately powered to answer the question of whether or not a narrow

surgical resection margin in early breast cancer patients treated with BCT results in an increase in local recurrence.

5.2.5.3 Hill criteria

Acknowledging the limitations of using a set of criteria for causal inference it is still worth examining this study in that light. This study will be examined from the perspective of each of the nine Hill criteria.(238, 240)

The first criterion is that of **temporal relationship.** Clearly for a factor to cause a disease state exposure to that factor must have occurred before the disease developed. It is evident that this criterion has been met in so much as any local recurrence occurs after the initial treatment has been completed.

The second criterion is that of the **strength of the association**. In this study there was no association noted between local recurrence and narrow resection margins.

The third criterion is that there is a **dose-response relationship.** If there was an association between the extent of surgical resection margin and local recurrence it would be logical that there would be some form of dose-response relationship, ie. the greater the margin of resection the less the local recurrence rate. In fact there are some who advocate wider and wider margins based on the assumption that such a relationship exists. One study examined in the literature review demonstrated an association between local recurrence and tumor volume near the resected margin. This one study, more so than the various re-excision studies, lends credence to some sort of dose-response curve but has not been validated in other studies. The extreme end of this position in fact would be a return to a mastectomy as the standard of care, which is unlikely to be accepted. There is adequate evidence to demonstrate an increasing range of local recurrence between grossly positive and focally positive lumpectomy specimens treated with BCS supported by re-excision data that shows greater residual cancer in grossly positive re-

excised lumpectomy specimens compared with focally positive re-excised lumpectomy specimens. It is a large step in logic to assume that a continuum exists from there to closely excised lumpectomies. Especially given that during the time frame of this and many of the studies in the literature a lumpectomy was often both a diagnostic and therapeutic tool (as opposed to the current practice where a core biopsy confirms the diagnosis most of the time in advance of the lumpectomy). Given that only about 20% of all wire guided biopsies were positive for cancer it was not that unusual for a lumpectomy to cut through tumor and result in a positive margin. In those cases the surgeon was intending to have a further discussion with the patient and plan further surgery based on that discussion. The continuum argument can only be considered if the initial lumpectomy specimen was always intended to be therapeutic.

Replication of findings is the fourth criteria. Even amongst the non-experimental studies the vast majority do not show a difference in local recurrence between narrow surgical resection margins and wider ones so long as there was no tumor at the inked resection margin.

Biological plausibility is the fifth of Hill's criteria. The fact that positive margins were clearly associated with an increased local recurrence combined with the re-excision studies demonstrating residual tumor in specimens that initially had a surgical resection margin less than or equal to 2 mm. undoubtedly was the impetus for the hypothesis that narrow margins might be associated with an increase in local recurrence. This hypothesis fails to take into consideration that BCT includes adjuvant radiation therapy. The appropriate question to ask is what is the amount of breast tissue that one has to remove that will allow for the adjuvant radiation treatment to deal with the residual tumor burden.

Consideration of alternate explanations and cessation of exposure and specificity of association, Hill's sixth and seventh and ninth criteria, are not applicable as there is no association noted.

Consistency with other knowledge while an important criterion that is worth exploring there is no other information that can be brought to bear on the subject that gives further illumination.

5.2.5.4 Study strengths

There are several strengths of this study above and beyond the study design and power issues discussed already. All histology other than invasive duct carcinoma were excluded, allowing us to confidently say that we are comparing like cases. Schwartz's textbook of surgery says that only 80% of all breast cancers are infiltrating duct carcinomas with the remainder largely being better prognosis tumors. All cases where the original pathology was not measured or a re-excision carried out with no residual tumor underwent independent pathologic review. There were almost no cases lost to follow-up. A multivariable regression analysis took into consideration other variables that have been associated with local recurrence.

5.2.5.5 Study limitations

The limitations of this study are primarily those inherent to a retrospective cohort study. In addition the single institutional nature of this study should be considered a limitation. Specifically, the pathologic review was limited to those cases where the margins could not be determined due to the practicality and cost of conducting a pathologic review of all cases. Other than margin status itself the limited pathologic review could have an impact on two other pathologic issues. Both of these issues could be of significant importance. First of these is the histologic type. There are distinct histologic categories that are in current practice that were not recognized during the timeframe that this study was conducted—particularly the triple negative or basaloid tumor (which is in reality a hybrid of histologic characteristics frequently found in

one of the genetic classifications) and the micropapillary tumor. Both of these are associated with a poorer prognosis. While uncommon we do not know the distribution of these two tumor histologies between the groups. Second, the presence or absence of DCIS and its extent (variables associated with local recurrence), may not be accurately categorized. In our study 614/825 (74.4%) of the cases had no mention of DCIS, 151/825 (18.3%) had focal DCIS, and 60/825 (7.3%) had extensive DCIS recorded. Prior to 1998 it was not routine to report the extent of DCIS in the pathology report. Moreover the presence and extent of DCIS at the tumor resection margin was not routinely reported and is a variable that was not considered in this study.

It is noted that the local recurrence rate in this study is higher than is seen in some current series. The literature shows a local recurrence rate with BCT to range from 3 to 20%.(61, 84, 191) In the study period used there was limited use of radiation boost (126/825 or 15.3%), which has since been demonstrated to decrease local recurrence significantly(72). Likewise literature shows that chemotherapy use is associated with a lower incidence of local recurrence (70, 230, 231) and the use of chemotherapy in the study period was lower than is current practice (only 377 or 45.7% of the entire group had some form of chemotherapy or hormonal therapy). Furthermore the follow-up in this study is a minimum of 10 years, which is significantly longer than most series. Local recurrence has been shown to continue to increase over time. A study with a longer follow-up period would be expected to have a higher local recurrence rate. These factors combined likely contribute to explaining the higher rate of local recurrence identified in this series compared with the current reality.

During the study period compression of wire guided specimens in diagnostic imaging were routinely performed, a practice that is no longer the case at least in Saskatoon. The extent to which these compressions distorted the surgical margin is unclear.

5.2.5.5 Evidence based medicine perspective

Prior to this study the level of evidence for the question of whether or not a narrow surgical resection margin is associated with an increase in local recurrence was level 4 (case-series and poor quality cohort or case-control studies). This retrospective cohort study elevates that to Level 3b

5.2.5.6 The "why not re-excise" argument

The suggestion made by some that, in the absence of convincing evidence to the contrary, one should do whatever possible to avoid local recurrence deserves attention. While not explicitly stated this opinion likely explains in the wide range of what practitioners consider to be an acceptable surgical resection margin in BCT done for early stage breast cancer.(61, 242)

Given that there is evidence that microscopic malignancy is left behind when a surgical resection margin is negative but less than or equal to 2 mm it might be tempting to suggest that this subgroup should have the "do more if in doubt" adage applied. To do so without consideration of the costs of that action fails to consider all facets of the decision being made. Not only are there clear physiological and financial costs to doing so the study done for this thesis raises the possibility that re-excision for a narrow margin might actually be associated with a greater risk of local recurrence, the exact opposite of what it is hoping to achieve. This study was not designed to address this unexpected finding but it is not the first time the possibility has been considered. In 1985 Papaioannou entertained the hypothesis that increasingly intensive locoregional treatment of breast cancer may promote recurrence through a variety of local, regional and systemic tumor-promoting mechanisms.(243) Menes et al. concluded that "the risk

of local recurrence after breast conservation for breast cancer increases progressively with the number of re-excisions needed to achieve clear margins" while O'Sullivan et al concluded exactly the opposite.(163, 164) There are several possible explanations for the observation made in this study. It is possible that there is a selection bias (surgeons selected cases more likely to recur locally for re-excision), there might be something negative about re-excision that makes local recurrence more likely (change in the local environment that makes radiation less effective, spread of tumor cells or poor orientation at the first surgery with a result of the re-excision being inadequate) or re-excision results in a delay in receiving adjuvant radiation which in turn results in an increase in local recurrence. The variables included in our multivariable analysis do not identify a particular variable that might have contributed to selection bias but does not eliminate this as a possibility. A delay in receiving adjuvant therapy is variably associated with increased local recurrence rates.(74, 78, 120) Therefore, time to receiving adjuvant therapy was added into the multivariable full model to assess this possibility. Time to treatment using cut offs of 6, 8, 10 or 12 weeks did not significantly affect local recurrence in any of the margin groupings.

5.2.5.7 Local recurrence rates—are they changing?

There is evidence that local recurrence rates are declining, likely for a variety of reasons.(244) The five year local recurrence rate in a recently published study of women treated with BCT between 1998 and 2002 showed a local recurrence rate of 3.1%. Earlier studies demonstrated local recurrence rates more in the 8% range. If this observation is true it is worth considering why that might be. There has been some speculation (and evidence) that the decrease in local recurrence rates can at least partially be attributed to the increase in specialization, with more patients receiving what is considered to be the standard of care.(244) An examination of the United States Surveillance Epidemiology and End Results (SEER) population based data shows that the proportion of women over the age of 65 with a diagnosis of breast cancer has gone from

37% in 1973 to 46.7% in 1995.(244) Older age is known to be associated with a better prognosis both in terms of survival and in terms of local recurrence in BCT. Therefore, even though the mechanism for the age association is not completely clear, one can anticipate this increasing age demographic alone to result in a decrease in the overall incidence of local recurrence in more current series of women undergoing BCT in early stage breast cancer. It is now common practice to treat node negative women with early stage breast cancer with systemic adjuvant therapy, either with endocrine therapy or cytotoxic chemotherapy. This was not the case during the time period of the study conducted for this thesis. In 1992 the Early Breast Cancer Trialist's Collaborative Group (EBCTCG) published a systematic overview of adjuvant systemic therapy.(230) Subsequent to that an international conference on adjuvant therapy of primary breast cancer was held. The recommendation coming out of that conference was that "almost all patients with clinical and pathologic stage I and II breast cancer, regardless of age, menopausal, nodal, or receptor status, will benefit from some form of adjuvant chemotherapy and /or hormonal therapy both in terms of improved disease-free, as well as overall survival.(231) Tamoxifen daily for five years reduces local recurrence risk by about 50% in estrogen receptor positive patients. (70)This benefit is extended to post-menopausal women with the addition of aromatase inhibitors.(245) Chemotherapy reduces the risk of local recurrence by about 30%, a benefit that is age-dependent being higher in younger women.(70) Trastuzumab has been shown to reduce local recurrence in Her2 positive women.(246, 247) Moreover, as mentioned earlier, the addition of a boost of radiation to the tumor bed has been shown to reduce local recurrence in early stage breast cancer treated with BCT.(72)

5.2.5.8 Conclusion

Returning to the question posed at the beginning of this section of whether or not it is reasonable to conclude that the re-excision of lumpectomy in which there is a surgical resection

margin less than or equal to 2mm is unwarranted in breast conserving surgery one must consider all of the above discussion. Of the twenty-two articles in the literature there are none that are of an experimental design. After removing the four articles that were excluded from the review only one was of a quasi-experimental design, the case-control study by Hardy that was underpowered. Of the remaining seventeen pre-experimental studies the vast majority did not show an association between a narrow surgical resection margin and local recurrence in early stage breast cancer treated with BCT. Of those that did, one was the same data set reviewed at different points in time and all had methodological concerns (see literature review). The study conducted for this thesis adds the only quasi-experimental study looking at the role of narrow surgical resection margins in local recurrence amongst women with stage I and II breast cancer treated with BCT that is adequately powered to address the question fully. From an evidence based medicine perspective this study elevates the level of evidence that narrow margin lumpectomies do not require re-excision to level 3 evidence. In the absence of a high level of evidence other information needs to be considered in making a decision. Clearly the treatment of early staged breast cancer has changed since patients included in the study conducted for this thesis underwent their cancer treatment. Few of the cohort studied were offered a boost of radiation to the tumor bed and a relatively small percentage of them were given adjuvant systemic treatment, two important treatment options in common practice today. Finally, the observation that the group who had a wide margin by virtue of a re-excision raises the possibility that re-excision might even be negative with regards to local recurrence in early stage breast cancer. While it is freely accepted that this study does not address this in a manner that can be anything other than an observation, there is recent evidence that makes this observation plausible. Demicheli et al. address the implied (almost never articulated) theoretical basis for the observed beneficial effect

of adjuvant radiation.(248) They examine the current theory that radiation's beneficial effect is derived from direct tumor cell death in light of the evidence and conclude "the beneficial effect of RT could be attributed not only to tumour cell killing but also, and probably more importantly, to its ability to modify the wound microenvironment, making it less favourable for cancer cell growth and invasion". Their observations are congruent with the emerging theories of oncogenesis reviewed earlier. In particular the cancer stem cell theory and the tissue organizational field theory (TOFT) have in common concepts about the tumor micro-environment.

In view of all of the above it is more than reasonable to assert that the current retrospective cohort study in the context of the current environment of care provided to stage I and II breast cancer patients offers enough evidence to end the current practice of re-excision of negative but narrow (less than or equal to 2 mm) surgical resection margin in these patients. Not only do such re-excision not offer any obvious advantage from a local recurrence perspective they could potentially be deleterious.

CHAPTER 6
CONCLUSIONS

In conclusion this retrospective cohort study confirms that a narrow (less than or equal to 2 mm) surgical resection margin in early stage invasive duct carcinoma treated with breast conserving therapy does not result in an increase in local recurrence over a minimum 10 year follow-up and therefore does not warrant re-excision.

CHAPTER 7
FUTURE DIRECTIONS

7.1 Overview

Personalized care is considered by many to be the future of cancer care. The "holy grail" of personalized cancer care is to have the ability to predict who will benefit the most from which treatment. In the case of local treatment the desire would be to perform breast conserving surgery on those women who will not have problems with local recurrence and to perform a mastectomy on those who will. With a 6-10% local recurrence rate over 10 years in early stage breast cancer treated with BCT it is clear that our ability to accurately predict who needs which surgical treatment has still not been achieved. In fact the only exclusion criteria currently applied to the decision making around BCS is documented multifocality, large tumors in a women with a small breasts, inflammatory cancer and advanced carcinomas—for everyone else BCT is considered the standard of care despite the know local recurrence rate and potential for it to impact survival.

In the absence of clear predictive markers to guide practitioners in choosing who should have BCT and who should have a mastectomy one must ask if there is anything that might give the medical community that kind of knowledge in the future.

7.2 Genomic prediction

7.2.1 Introduction

Increasingly it is understood that cancer is not one heterogeneous disease making it probable that the tools that have been traditionally used to prognosticate, such as surgical margins, grading systems etc., may be inadequate to predict outcome. Molecular options of examining tumors have opened the possibility of a more accurate modality of prognostication.

7.2.2 DNA microarray and local recurrence

Gene expression profiles have captured the imagination of the cancer research community with the hope of being able to better select patients for care based on the genetic profile their particular tumor. In terms of this new modality's role in predicting local recurrence there have only been four publications to date. In 2006 Cheng et al. published a study whose purpose was to explore gene expression profiles that are associated with locoregional recurrence in breast cancer after mastectomy.(249) They concluded that gene expression profiles do predict for locoregional recurrence and can be used to select patients for post mastectomy radiation therapy. In the same year Nuyten et al. published a paper indicating that gene expression profiling can identify subgroups of patients at increased risk of developing a local recurrence after breast-conserving therapy.(250) Ignatiadis and Desmedt offer their opinion that genomic profiling in combination with clinicopathologic parameters will be able to discern more accurately those who will recur but does not move that hope any closer to reality.(251) Most recently Nimeus-Malmstrom et al. report a "highly distinct gene expression profile from patients developing local recurrence after breast-conservation surgery despite radiotherapy."(252) Undoubtedly there will be further reports in the literature in the future that will clarify the role of gene expression profiling in predicting local recurrence in early stage breast cancer patients with a view to helping make the decision of whether to consider BCS or not.

7.2.3 MiRNA and local recurrence

7.2.3.1 Introduction

MicroRNA (miRNA) analysis of tumors is a more recent molecular test that is more useful for paraffin embedded tissue samples that have been stored for longer periods of time. The possibility that miRNA techniques might provide predictive and prognostic information regarding local recurrence in early stage breast cancer, thus allowing for retrospective studies to

be performed on tissue samples that have been stored for years, was explored in a pilot study conducted as a "future directions" component this thesis work.

miRNAs are small, non-coding RNA molecules, involved in post-transcriptional gene regulation.(218) They have been found to be more stable than messenger RNA (miRNA) in FFPE tissues. It is thought that given the size and stability of miRNAs, they are less affected by autolysis, cross-linking and fragmentation that occurs with DNA and miRNA during formalin fixation and storage.(253, 254) Previous studies have shown that miRNA in FFPE samples in colorectal cancer dating back ten years remain stable over that time frame and provide promising targets for research into cancer biomarkers.(254)

miRNAs are typically 21-23 nucleotides in length and function through the translational inhibition or degradation of miRNA within the cytoplasm.(255) This is accomplished by complimentary binding of the miRNAs to the 3' untranslated region of the target miRNA in a ribonucleoprotein complex known as the RNA-induced silence complex (RISC). Depending on the degree of base pairing, the target miRNA will either be degraded, if tightly bound, or protein synthesis will be impaired, if loosely bound. Each miRNA is capable of targeting approximately 200 different miRNA s and potentially one third of human miRNA s may be regulated in such a manner.(256)

miRNAs regulate various cell cycle processes, and as such can act in both an oncogenic or tumor suppressor role depending on the genes targeted.(257) In breast cancer miRNAs have been shown to be involved in self renewal, tumorigenicity, invasion and metastasis.(258-261) The impact of miRNAs on breast cancer local recurrence has yet to be studied.

7.2.3.2 Study question

The question being addressed by this study was whether or not miRNA microarray analysis will contribute in a meaningful way to the prediction of local recurrence in early stage breast cancer treated with BCT. The attraction of this technology is that miRNA have significantly greater stability in FFPE tissue samples. Because of this miRNA microarray analysis can be performed on FFPE samples that have been in storage for a long enough period of time. Consequently quick results can be achieved unlike DNA microarray studies, which need to be conducted more or less prospectively given the instability of the samples in FFPE tissue.

7.2.3.3 Study objectives

Given the very early stages of research with this modality the primary question of the utility of miRNA microarray analysis was broken down into three questions. The first of these was to determine whether or not FFPE tissue samples that have been stored for 10 years or longer are still useful. The second question was if a miRNA profile emerged as a candidate for further study. Finally, even though the study was conducted using a very small sample size it was thought worthwhile to see if a miRNA emerged that might predict local recurrence in early stage breast cancer treated with BCT.

7.2.3.4 Hypothesis

The hypothesis is that miRNA microarray analysis has potential as a research tool that might lead to the identification of candidate miRNA, which might in turn be predictive of local recurrence in early stage breast cancer.

7.2.3.5 The study design

A small convenience sample of 4 locally recurrent specimens and 4 non-recurrent specimens from the larger cohort that comprised the local recurrence in early stage breast carcinoma with narrow margin study was chosen for analysis. The details of the patient

characteristics of these eight patients are presented in table 7.1. Tissue blocks that had an adequate amount of tumor in the paraffin embedded block were selected in order to ensure adequate tumor sampling. Sample age varied from ten to fourteen years. These eight specimens were then be subjected to a hierarchical miRNA micro array analysis.

Table 7.1 Details of cases examined

ID	LR	DATE OF DX	# NODES	SIZE	MARGIN	ER	PR
1	no	Aug-99	0	3 cm	narrow	+	+
2	no	Sep-97	0	1.2 cm	narrow	+	+
3	no	Oct-00	0	2.3 cm	wide	+	+
4	yes	Jun-00	9	4 cm	narrow	+	+
5	no	May-99	0	1.5 cm	narrow	+	+
6	yes	Mar-95	0	1.3 cm	narrow	+	+
7	yes	Nov-96	3	1.6 cm	narrow	+	+
8	yes	Aug-96	2	2.5 cm	wide	+	+

Two one millimeter core sections of tumor were obtained from each of eight FFPE tissue samples. In order to assure that the core samples contain malignant tissue a single H&E slide was cut and the tumor outlined on the slide by an expert breast pathologist. The cores were taken from tissue outlined on the H&E slide.

The first piece of information that was determined was the amount of intact miRNA extracted from the core biopsies. Given the age of the samples it was important to assess whether or not the tissue sampled has enough intact miRNA. Total RNA was isolated from the FFPE samples using the RecoverAll Total Nucleic Acid Isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions.(253) Agilent MicroRNA V3 arrays, which detect 866 human

miRs, was used for profiling. For each sample the cores were deparaffined with xylene, washed twice with ethanol and digested with protease at 50 °C for three hours. The lysate was then passed through a filter cartridge and the RNA eluted in 60 μl water. The amount of RNA protein was diluted so that each of the eight samples contains about 50 nanograms per milliliter of solution. The total RNA amount was assessed for purity using spectrophotometry.

After assuring that there was adequate miRNA for assessment the next part of the study was to perform a supervised and unsupervised hierarchical clustering analysis using microarray technology. A microarray is an orderly array of miRNA attached to a solid support. In this study 866 know human miRNA were used in the array. The miRNAs were labeled using the Agilent miRNA complete labeling and hybridisation kit. Approximately 100 nanograms of total RNA from each sample were dephosphorylated and ligated with pCp-Cy3. The labeled RNA was then hybridized to the Agilent miRNA arrays at 55°C for 20 hours. The arrays were then scanned with the Agilent DNA Microarray scanner. The signals quantified using the Agilent Feature Extraction software which has the ability to semi-automatically identify probe spots and present the raw data on the fluorescence intensities obtained for each individual pixel within the spot.

Finally a supervised hierarchical clustering algorithm was applied comparing those tumors with local recurrence with those without local recurrence to see if there was any significantly different patterns or if there are any miRNA identified that might be used as a candidate miRNA in future studies.

It is clearly understood that with a sample of only eight cases this was but an early pilot study. Provided there is adequate extraction of intact miRNA to perform the mircoarry analysis and candidate miRNAs emerge for consideration further analysis on a larger set of tumors would be

warranted, which might in turn lead eventually to validation studies. This further work is beyond the scope of this PhD thesis.

7.2.3.6 Ethics approval

Ethics approval was obtained from the Biomedical Research Ethics Board at the University of Saskatchewan (Bio-REB # 09-14).

7.2.3.7 Statistical analysis

Data analysis was performed using Agilent Gene Spring GX software version 10.5.1.1. Unsupervised clustering was performed on the raw data. P values were calculated using the student t test. A p value of <0.05 was considered statistically significant.

7.2.3.8 Results

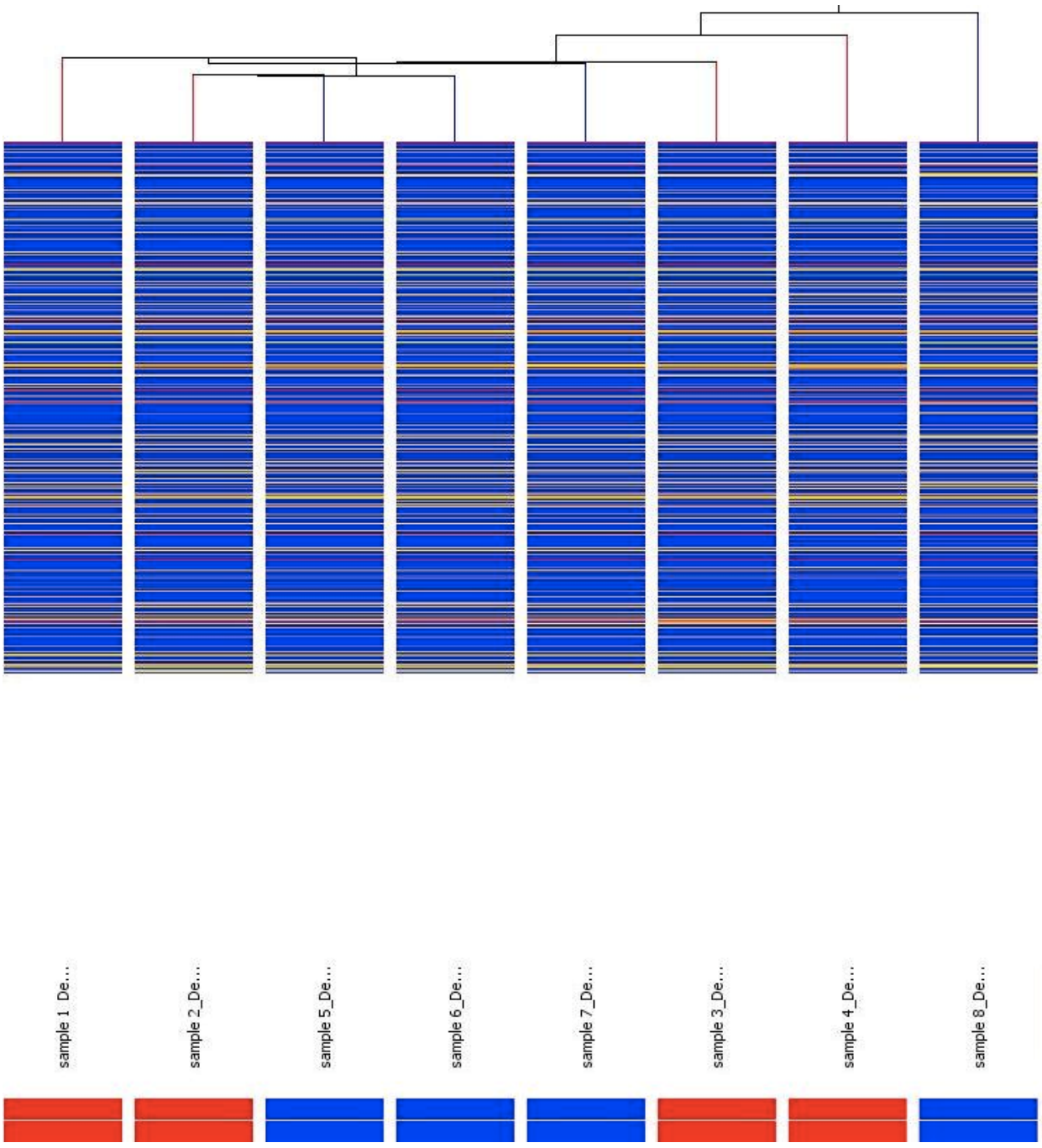
7.2.3.8.1 Quality of miRNA extracted

In order to provide reliable data through microarray testing one must ensure that the samples provide sufficient miRNAs for analysis. The quality and quantity of miRNA isolated from the FFPE samples was shown to be adequate for accurate microarray analysis. Initial analysis using spectrophotometry to assess for the quality and purity of total RNA isolates provided 260/280 and 260/230 ratios indicative of high purity RNA samples. Quality control analysis of the microarray data was performed using quality metrics scores, which assesses various quality parameters including background noise, population outliers, signal uniformity and intensity. For each sample the values for each parameter were found to lie within the standards indicative of adequate quality.

7.2.3.8.2 Unsupervised hierarchical clustering analysis and Candidate miRNAs

The basis of unsupervised clustering is to group tumors into homogenous classes based on gene expression profiles. 866 miRs were represented on the Agilent MicroRNA V3 arrays. Tumor samples were analyzed in an unsupervised manner so as to examine the intrinsic structure of the dataset and results were summarized in a dendrogram (Figure 7.1). Unsupervised hierarchical clustering failed to identify any miRNA profile amongst the eight samples studied.

Figure 7.1 Supervised hierarchical clustering of miRNA expression data with mapping of miRNA expression profile.



7.2.3.8.3 Supervised Analysis

The data was further analyzed to assess for individual candidate genes that displayed expression changes between the recurrent versus non-recurrent groups. This was performed using Agilent Gene Spring GX. No miRNAs passed significance analysis, however there were a number of miRNAs which did exhibit expression differences between the two groups. The data was analyzed for miRNAs with fold changes ≥ 2, 44 miRNAs were found to meet this criterion, 8 of which had fold changes ≥ 3 (Table 7.2).

Table 7.2: Candidate miRs with fold change greater then 3

miRNA	**Fold Change**	**Regulation (Recurrence vs No Local Recurrence)**
miRNA-451	6.541835	Up
miRNA-363	5.9384456	Down
miRNA-205	3.9676561	Down
miRNA-203	3.8479598	Up
miRNA-224	3.4443598	Up
miRNA-7	3.406654	Up
miRNA-18a	3.0821824	Up
miRNA-1254	3.0308013	Down

7.2.3.9 Discussion

miRNAs are known to be involved the regulation of various cancers including breast cancer. To date the possibility of miRNA profiling predicting local recurrence in early stage breast cancer has not been explored and as such offer a novel area of investigation. The pilot study conducted for this thesis analyzed the expression of 866 miRNAs in four locally recurrent breast cancers and four non-recurrent breast cancers. Despite the age of the samples (ranging from 10-14 years) the quantity and quality of intact miRNAs from the PFFE samples was acceptable, supporting the use of miRNA microarray profiling in the study of archival FFPE tissues samples stored for up to 14 years. Not surprising given the small sample size the two groups failed to cluster in a significant manner but 44 miRNAs were differentially expressed, 8 of which had at least a 3 fold change. These eight candidate miRNA warrant further investigation with one in particular deserving of particular attention.

Of significant interest in local recurrence, miRNA-205 is known to be down regulated in breast cancer, and is thought to play a tumor suppressor role.(261) In our study it was shown to be down regulated in recurrent cancers by 4.0 fold compared to non recurrent cancers. It is known to hinder invasion and metastasis in breast cancer through its inhibition of Erb-B3 and VEGF-A.(262) Of particular interest in local recurrence, miR-205 has been shown to be significantly associated with locoregional recurrence of head and neck squamous cell carcinoma, independent of disease severity at diagnosis and treatment.(263) These findings certainly point to miR-205 as a suitable candidate for further investigations into its role with breast cancer local recurrence.

7.2.3.10 Conclusions

miRNA expression profiling can be done successfully on FFPE tissue samples as old at 14 years. The amount of intact miRNA recovered is acceptable. The value of the candidate miRNA-205 in particular warrants further study on a larger sample of breast specimens.

7.3 Randomized controlled trial in the modern setting

7.3.1 Introduction

While the study done to for this thesis does not demonstrate an increased local recurrence rate with a narrow (less than or equal to 2 mm) but negative surgical resection margin in early stage breast cancer treated with BCT the modern management of early stage cancer has changed significantly from the time that the data from this study was collected. As mentioned earlier the retrospective and single institutional nature of the study also carries with it limitations. It would be ideal to construct a prospective randomized study to confirm the observations of this thesis work in the modern setting.

7.3.2 Potential study design

I would suggest a study design in which all patients who present with an early stage breast cancer (stage I or II) who are treated with lumpectomy, registered with the Saskatoon Cancer Agency, and considered for post-operative adjuvant radiation therapy be considered eligible for enrollment. Ideally similar patients registered in other cancer centers would also be registered. Those patients who have an initial narrow (less than 2 mm) surgical resection margin would randomly be assigned to one of three groups following appropriate consent—re-excision to obtain a wide surgical resection margin, no re-excision but a boost of radiation of 1,600 Gy or no re-excision and no radiation boost. Adjuvant radiation and chemotherapy would be administered according to normal

protocol. Pathologic reporting would be synoptic according to current guidelines. In addition all specimens would undergo genetic assessment with both DNA microarray and miRNA microarray as would any local recurrences in order to be able to determine whether or not the local recurrence is a true recurrence or a new primary. Information on the other variables of interest as determined by the literature review conducted for this thesis would be also be collected.

7.2.4 Sample size

Given that a the modern local recurrence rate is lower (about half) than it was during the time when the data was collected for the study conducted for this thesis the calculated number of patients required in each arm would be 400.

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APPENDIX A

PUBLISHED ARTICLE *JOURNAL OF SURGICAL ONCOLOGY,* MARCH 2011

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Predicting Local Recurrence Following Breast-Conserving Therapy for Early Stage Breast Cancer: The Significance of a Narrow (≤2 mm) Surgical Resection Margin

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Background and Objectives: Controversy continues over the extent of surgical resection margin required to minimize the risk of local recurrence (LR) in breast-conserving therapy (BCT) for early stage breast cancer. This study explores whether or not a narrow (≤2 mm) but negative resection margin affects LR.
Methods: All patients registered at the Saskatoon Cancer Center between January 1, 1991 and December 31, 2000 with a diagnosis of early stage invasive duct carcinoma treated with BCT were examined. All charts and pathology reports were reviewed with a review of the pathology for all cases where the resection margin was unclear in the original report. Other factors known or thought to effect LR (age, radiation boost, grade, extensive DCIS, ER/PR receptor status) were considered in the statistical analysis.
Results: Amongst the 200 narrow margin cases 19 LR were detected (19/201 = 9.5%) while 52 LR were detected in the 491 wide margin cases (52/491 = 10.6%). This difference was not statistically significant.
Conclusions: A narrow (≤2 mm) surgical resection margin does not result in an increase in LR compared to a surgical resection margin >2 mm in BCT for early stage duct carcinoma and does not warrant re-excision.
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KEY WORDS: **breast-conserving therapy; neoplasm recurrence; local**

INTRODUCTION

Beginning with Fisher's seminal publication in 1985 [1], there have been many publications, including 10 long-term randomized controlled trials [2–11], demonstrating that breast conserving surgery (BCS) has equal survival compared with a modified radical mastectomy. Breast-conserving therapy (BCT) is, however, known to be associated with a greater incidence of LR compared to mastectomy [7,12–17]. There is good evidence that a positive surgical resection margin is associated with increased LR and re-excision is warranted in that situation [18–27]. Fisher defined negative margins as, "no ink at the surgical resection margin" but controversy remains as to whether or not that definition is adequate to minimize the rate of the local recurrence (LR) in BCT [20,28]. Studies have demonstrated that specimens with an initial narrow (≤2 mm) but negative margin of resection, when re-excised, frequently had residual tumor in the re-excised specimens compared to re-excised specimens with an original resection margins >2 mm (where there was rarely residual tumor found) [29–31]. It would seem that it is these studies that have led to the recommendation commonly found in practice guidelines that narrow margin tumors should be re-excised prior to adjuvant radiation therapy.

Re-excision comes at a cost. Delays in starting adjuvant therapy, increased wound infection rates, poorer cosmesis, and loss in confidence are all potential effects of re-excision not to mention the increased economic cost associated with a second surgery. It would therefore be prudent to avoid unnecessary re-excision unless required for sound oncologic reasons.

The purpose of this article is to determine if a narrow (≤2 mm) but negative surgical resection margin in BCT done for early stage breast cancer results in an increased LR rate.

MATERIALS AND METHODS

Ethics approval was obtained from the University of Saskatchewan Health Research Ethics Board as well as the Saskatchewan Cancer Agency. All patients treated at the Saskatoon Cancer Agency during the years of 1991–2001 with a diagnosis of invasive duct carcinoma of the breast, which were stage, I or II, and who had BCT as their method of treatment were included in this study. This time frame was chosen for three reasons. First, we have a minimum 10 years of follow-up by ending our data collection in 2001. Second, the sample size calculation described below, combined with an initial review of the number of cases being registered at our Cancer Center led us to calculate a need for 10 years worth of patient data being required. Finally, during the time period chosen it was not standard practice to re-excise narrow margin specimens, hence we assumed that we would be able to accrue the required number of narrow margin cases. All of these charts were reviewed to determine whether the inclusion criteria were met. Patients who went on to have a mastectomy, who had a different diagnosis, who had a more advanced stage, who had a positive resection margin, or who did not have radiation treatment were excluded from further analysis. For the remainder the last date of follow-up or LR was recorded as the primary endpoint.

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LR was defined as an ipsilateral breast recurrence of the same histology as recommended in the literature [32–35]. Nodal recurrence, metastatic disease, tumors of different histology from the primary and skin only recurrence were not considered LR.

The margin status was determined by reviewing the pathology report, which was issued based on formalin fixed paraffin embedded specimen analysis. In cases where the pathologist had recorded the margin this was taken to be accurate and valid. In cases where there was a re-excision and there was no residual tumor in the re-excised specimen the margin was considered to be >2 mm and was recorded as "wide margin on the basis of re-excision." In cases where the margin status could not be determined from the chart the slides were retrieved and independently reviewed. In those cases where the pathologic review could not be determined this was recorded as being unable to assess.

Based on a review of literature other variables considered to be associated with LR included receptor status (both estrogen and progesterone), tumor grade, tumor size, age, extensive duct carcinoma in situ, radiation boost, and adjuvant chemo or hormonal therapy. Data on these variables were collected and analyzed to assess their contribution.

A sample size calculation determined that 200 patients would be required in each arm of the study (narrow margin and wide margin) to detect a 50% difference in LR assuming a baseline 10% incidence of LR over 10 years.

The statistical analysis was done using SPSS 17 to perform the logistic regression analysis using standard statistical methods.

RESULTS

In the 10-year period 3,960 patients were registered with the Saskatoon Cancer Agency with a diagnosis of breast cancer. Of these 2,872 were either stage III or IV, had a mastectomy as their initial surgical procedure, had a diagnosis other that invasive duct carcinoma or failed to receive adjuvant radiation. The charts were reviewed in all of the remaining 1,088 patients.

Of the charts reviewed a further 263 were excluded. The majority of these were excluded because they went on to a mastectomy after their initial segmental resection. Other reasons for exclusion included: misclassification (wrong diagnosis, advanced stage, incomplete or no adjuvant radiation) or inability to locate the chart in 25. In the remaining 825 charts there were 323 in which the surgical margin was measured in the original pathology report, 239 in which there was a re-excision in which no residual tumor was detected, and 263 cases in which the report was unclear and required an independent pathologic review. Amongst the 263 reviewed cases 124 were unable to be assessed either because the slides were lost, the margin was never marked, or the stain had faded to an extent that it was impossible to tell. Table I lists the various margin categories broken down into LR or not.

A logistic regression analysis comparing the 201 narrow margin cases with the 500 wide margin cases showed no statistical difference in LR rate ($P = 0.499$). When broken down into measured wide margins and wide margin by virtue of being re-excised, however, the re-excised group had a statistically greater change of LR ($P = 0.02$) (Table II).

TABLE I. Local Recurrence Rates for Narrow and Various Wide Excision Groups

	Local recurrence	Total	% Local recurrence
Narrow margin	19	201	9.5
<1 mm	5	47	10.6
1–2 mm	14	154	9.1
Wide margin	56	500	11.2
Measured	21	261	8
By re-excision	35	239	14.6
Total	75	701	

TABLE II. Statistical Analysis of Local Recurrence by Margin Status

Margin	OR (CI)	*P*-value
Narrow vs. wide combined	1.208 (0.698–2.090)	0.499
Narrow	0.608 (0.336–1.101)	0.101
Measured wide	0.510 (0.288–0.904)	0.021
Re-excised wide	1	
Narrow	1	
Measured wide	0.838 (0.48–1.605)	0.594
Re-excised wide	1.643 (0.908–2.974)	0.101

Univariate analysis was performed for each of the potentially important independent variables identified in the literature as associated with LR (age < 40, grade, adjuvant chemo or hormonal therapy, estrogen and progesterone receptor status, radiation boost, tumor size, and the

TABLE III. Univariate Analysis of Variables

Variable	OR (CI)	*P*-value
Size (T1 vs. T2)	1.401 (0.844–2.325)	0.139
Size in cm		
<1 cm	1	
1–2 cm	0.995 (0.599–1.653)	0.948
2.001–3 cm	1.16 (0.589–2.285)	0.667
3.001–4 cm	2.011 (0.811–4.988)	0.131
4.001–5 cm	2.463 (0.488–12.419)	0.275
<1 cm	0.406 (0.081–2.047)	0.275
1–2 cm	0.404 (0.081–2.012)	0.268
2.001–3 cm	0.471 (0.089–2.497)	0.376
3.001–4 cm	0.817 (0.139–4.813)	0.823
4.001–5 cm	1	
Grade 1	0.406 (0.214–0.770)	0.006
Grade 2	0.679 (0.393–1.171)	0.164
Grade 3	1	
Grade 1	1	
Grade 2	1.673 (0.929–3.012)	0.086
Grade 3	2.466 (1.299–4.680)	0.006
Margin of resection		
Narrow	0.608 (0.336–1.101)	0.101
Wide	0.510 (0.288–0.904)	0.021
Wide by re-excision	1	
Narrow	1	
Wide	0.838 (0.438–1.605)	0.594
Wide by re-excision	1.643 (0.908–2.974)	0.101
Extensive DCIS (yes/no)	0.596 (0.291–1.220)	0.157
DCIS status		
None	0.648 (0.314–1.336)	0.24
Focal	0.393 (0.157–0.981)	0.045
Extensive	1	
None	1	
Focal	0.606 (0.313–1.175)	0.138
Extensive	1.543 (0.749–3.180)	0.24
Nodal status (pos./neg.)	1.458 (0.908–2.342)	0.119
ER status (pos./neg.)	1.003 (0.991–1.016)	0.595
PR status (pos./neg.)	1.002 (0.989–1.014)	0.805
Radiation boost (yes/no)	1.360 (0.702–2.634)	0.362
Chemo/hormonal adjuvant therapy (yes/no)	1.087 (0.701–1.686)	0.708
Chemotherapy groups		
Hormonal	0.388 (0.155–0.971)	0.043
AC	0.671 (0.211–2.130)	0.499
CMF	0.490 (0.174–1.379)	0.177
Other	1	
Hormonal	1	
AC	1.728 (0.639–4.668)	0.281
CMF	1.262 (0.540–2.954)	0.59
Other	2.574 (1.030–6.437)	0.043
Age (≤40/>40)	2.821 (1.371–5.804)	0.005

TABLE IV. Details of the Multivariable Logistic Regression Analysis for Local Recurrence in Early Stage Breast Cancer

Variable	Multivariable OR (CI)	*P*-value
Narrow margin vs. wide margin measured margin	0.689 (0.334–1.423)	0.314
Narrow margin vs. wide re-excised margin	1.576 (0.815–3.044)	0.176
Wide measured margin vs. wide re-excised margin	0.437 (0.232–0.826)	**0.011**
Age ≤40	1.736 (0.672–4.355)	0.24
Grade 2 vs. grade 1	1.72 (0.871–3.394)	0.118
Grade 3 vs. grade 1	2.832 (1.298–6.178)	**0.009**
Grade 2 vs. grade 3	0.607 (0.323–1.143)	0.122
Extensive DCIS	1.565 (0.608–4.027)	0.353
Positive nodal status	1.393 (0.790–2.457)	0.252
Adjuvant chemotherapy or hormonal therapy given	1.478 (0.814–2.683)	0.199
T2 vs. T1 tumor size	1.28 (0.681–2.406)	0.443
Radiation boost given	1.407 (0.640–3.093)	0.395

The bold indicates the two variables with a P value <0.05.

presence of extensive duct carcinoma in situ). Only re-excised wide margin, high grade and age <40 were found to be statistically significant in that univariate analysis (Table III). The full multivariable model presented in Table IV did significantly predict LR. Each variable was subsequently fit into a model testing for interaction between it and the main variable of interest (margin status) and no significant interaction effects were identified. Finally a Kaplan–Meier survival curve was calculated and is presented in Figure 1 rather than Table V. This shows no statistically significant difference between the narrow and wide margin groups for LR beyond 10 years

DISCUSSION

This study confirms that a narrow but negative surgical resection margins in early stage invasive duct carcinoma treated with BCT does not result in a greater likelihood of LR compared with wider resection margins (either wider margins by measurement combined with re-excised wider margins, wider margin by measurement alone, or wider margin by re-excision alone). It would follow that re-excision of narrow margin segmental resections is therefore unwarranted. We also noted that re-excised but wide margin tumors had a significantly greater chance of developing a LR. This study was not designed to address this unexpected finding. There are several possible explanations for this observation. It is possible that there is a selection bias (surgeons selected cases more likely to recur locally for re-excision), there might be something negative about re-excision that makes LR more likely (change in the local environment that makes radiation less effective, spread of tumor cells or poor orientation at the first surgery with a result of the re-excision being inadequate) or re-excision results in a delay in receiving adjuvant radiation which in turn results in an increase in LR. The variables included in our multivariable analysis do not identify a particular variable that might have contributed to selection bias but does not eliminate this as a possibility. A delay in receiving adjuvant therapy is associated with increased LR rates [36–38]. Therefore, time to receiving adjuvant therapy was added into the multivariable full model to assess this possibility. Time to treatment using cut offs of 6, 8, 10, or 12 weeks did not significantly affect LR in any of the margin groupings.

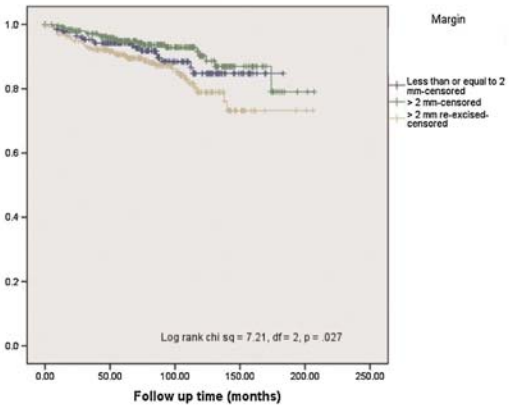

Fig. 1. Kaplan–Meier Curve demonstrating length of time to local recurrence.

In reviewing the literature there are two recent surveys demonstrating a wide range of what practitioners consider to be an acceptable surgical resection margin in BCT done for early stage breast cancer [28,39]. The British Columbia Cancer Agency website on this issue (last updated November 2004) says, ''Re-excision to obtain negative margins is recommended for patients with close or positive margin'' a position adopted in Saskatoon in recent years.

A review of the literature looking for studies examining LR and margin status revealed several studies that performed multivariate regression analysis of a variety of factors that might be associated with LR. In these studies a number of factors emerged as being associated with LR. Amongst these age less than 40 [40–47], and positive resection margin [18–27] were the most consistently associated. Narrow margins were associated with LR in 3 of 6 papers and other factors identified included extensive DCIS. The study by Hardy et al. [48] is the only one in the literature that explicitly examines the role of a narrow surgical resection margin in LR for early stage breast cancer. This case–control study, which showed no benefit to wider histologically negative margins, unfortunately had a number of methodological limitations.

The strengths of our study are several. We excluded all histologies other than invasive duct carcinoma allowing us to confidently say that we are comparing like cases. All cases where the original pathology was not measured or a re-excision carried out with no residual tumor underwent independent pathologic review. There were almost no cases lost to follow-up. A multivariable regression analysis took into consideration other variables associated with LR.

The limitations of our study are primarily those inherent to a retrospective cohort study. Specifically, the pathologic review was limited to those cases where the margins could not be determined. Consequently, other than the margin status itself, the presence or absence of DCIS and its extent (variables associated with LR), may not be accurately categorized. In our study 614/825 (74.4%) of the cases had no mention of DCIS, 151/825 (18.3%) had focal DCIS, and 60/825 (7.3%) had extensive DCIS recorded. Likewise we were not able to confirm the histologic diagnosis as they were not all reviewed. It is noted that the LR rate in this

study is higher than is seen in current series. The literature shows a LR rate with BCT to range from 8% to 20% [18,19,28]. In the study period used there was limited use of radiation boost (126/825 or 15.3%), which has since been demonstrated to decrease LR significantly [49]. Likewise literature shows that chemotherapy use is associated with a lower incidence of LR [18] and the use of chemotherapy in the study period was lower than is current practice (only 377 or 45.7% of the entire group had some form of chemotherapy or hormonal therapy). Both of these factors combined likely contribute to explaining the higher rate of LR identified in this series compared with the current reality.

CONCLUSIONS

In conclusion this retrospective cohort study confirms that a narrow (≤2 mm) surgical resection margin in early stage invasive duct carcinoma treated with BCT does not result in an increase in LR over a minimum 10-year follow-up and therefore does not warrant re-excision.

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APPENDIX B

CENTER FOR EVIDENCE BASED MEDICINE LEVELS UNIVERSITY OF OXFORD

Level 1A	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	1a SR (with homogeneity*)of RCTs SR (withhomogeneity*) of inception cohort studies; CDR† validated in different populations SR (with homogeneity*) of Level 1 diagnostic studies; CDR† with 1b studies from different clinical centres SR (with homogeneity*) of prospective cohort studies SR (with homogeneity*) of Level 1 economic studies
Level 1b	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	Individual RCT (with narrow Confidence Interval‡) Individual inception cohort study with > 80% follow-up; CDR† validated in asingle population Validating** cohort study with good††† reference standards; or CDR† tested within one clinical centre Prospective cohort study with good follow-up**** Analysis based on clinically sensible costs or alternatives; systematic review(s) of the evidence; and including multi-way sensitivity analyses
Level 1c	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	All or none§ All or none case series Absolute SpPins and SnNouns†† All or none case-series Absolute better-value or worse-value analyses ††††
Level 2a	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	SR (with homogeneity*) of cohort studies SR (withhomogeneity*) of either retrospective cohort studies or untreated control groups in RCTs SR (with homogeneity*) of Level >2 diagnostic studies SR (with homogeneity*) of 2b and better studies SR (withhomogeneity*) of Level >2 economic studies
Level 2b	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	Individual cohort study (including low quality RCT; e.g., <80% follow-up) Retrospective cohort study or follow-up of untreated control patients in an RCT; Derivation of CDR† or validated on split sample §§§ only Exploratory** cohort study with good††† reference standards; CDR† after derivation, or validated only on split-sample§§§ or databases Retrospective cohort study, or poor follow-up Analysis based on clinically sensible costs or alternatives; limited review(s) of the evidence, or single studies; and including multi-way sensitivity analyses
Level 2c	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	'Outcomes' Research; Ecological studies 'Outcomes' Research Ecological studies Audit or outcomes research
Level 3a	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	SR (with homogeneity*) of case-control studies SR (with homogeneity*) of 3b and better studies SR (with homogeneity*) of 3b and better studies SR (with homogeneity*) of 3b And better studies
Level 3b	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	Individual Case-Control Study Non-consecutive study; or without consistently applied reference standards Non-consecutive cohort study, or very limited population Analysis based on limited alternatives or costs, poor quality estimates of data, but including sensitivity analyses ‖incorporating clinically sensible variations.
Level 4	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	Case-series (and poor quality cohort and case-control studies§§) Case-series (and poor quality prognostic cohort studies***) Case-control study, poor or non-independent reference standard Case-series or superseded reference standards Analysis with no sensitivity analysis
Level 5	Therapy/Prevention, Aetiology/Harm Prognosis Diagnosis Differential diag/symptom prevalence Economic and decision analyses	Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles" Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles" Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles" Expert opinion without explicit critical appraisal, or based on physiology, bench research or "first principles" Expert opinion without explicit critical appraisal, or based on economic theory or "first principles"

Grades of Recommendation

A	consistent level 1 studies
B	consistent level 2 or 3 studies or extrapolations from level 1 studies
C	level 4 studies or extrapolations from level 2 or 3 studies
D	level 5 evidence or troublingly inconsistent or inconclusive studies of any level