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PRINCIPAL INVESTIGATOR: Carolyn Mountford, Ph.D.

CONTRACTING ORGANIZATION: University of Sydney
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Fine needle aspirates have been obtained from patients undergoing breast surgery and analysed by proton MR spectroscopy at 8.5 Tesla and results compared with histopathological analysis. The results of the blinded MR analysis were compared with currently employed diagnostic approaches used in assessment of breast lesions. In the study so far discrimination between invasive carcinoma (n=82) and normal or benign (n=106) or carcinoma *in situ* (n=17) gave a sensitivity and specificity for benign versus invasive breast cancer of 95% and 96% respectively. The data are now being reanalysed by a robust multivariate analysis procedure.

All carcinoma-*in-situ* samples with comedonecrosis or a microinvasive component (n=6) were ranked by MRS with invasive carcinoma while those with *in-situ* disease alone ranked with benign (n=11).

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Carolyn Mooney

PI - Signature **Date**

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INTRODUCTION

Recent improvement in breast cancer patient outcome is due to early diagnosis and effective management. The triage of mammography, clinical examination and fine needle aspiration biopsy is currently used to identify early breast cancers. Magnetic resonance imaging (MRI) has now been added to select women with breast abnormalities requiring biopsy. However, mammography and MRI are unable to distinguish cellular changes which correlate with the development of cancer and are unable to predict patient outcome. Fine needle aspiration cytology following mammography has reduced the incidence of unnecessary surgery but cannot predict at-risk status or tumour progression.

A new technology which could report on alterations to cellular chemistry and provide an adjunct to cytology would offer both independent and objective assessment of breast tissue. The potential then exists to identify an immediate or more distant predisposition towards breast cancer and thus screen, monitor and offer management protocols aimed at reducing tumour risk. Magnetic resonance spectroscopy offers this diagnostic modality and has been successfully developed to monitor tumour development and progression in other organs.

In the clinical situation where an established diagnosis of breast cancer has already been made, current predictors of patient outcome are of limited value. Despite methods of evaluation such as tumour typing and grading, surgico-histopathological staging, hormone and growth factor receptor status, genetic markers and ploidy, the reason for the variable clinical outcome in patients with established widespread metastatic disease remains unknown. The identification of biochemical markers both within primary and metastatic tumour deposits may be of great value in identifying patients who are likely to respond to specific therapeutic regimes.

This proposal is aims at developing magnetic resonance spectroscopy to be used as an adjunct to diagnostic histopathology. The MRS method offers the possibility of earlier diagnosis of cellular abnormalities in predisposed women, a precise documentation of the biological abnormalities of the cells, conclusive predictive value, clear distinction between cellular atypia, progressive and non-progressive carcinoma *in situ* and invasive cancer and the potential to determine patient outcome. These advantages over current technology warrant the thorough investigation of the application of magnetic resonance spectroscopy to breast pathology.

SUMMARY OF PROGRESS

To date 218 FNA have been obtained from patients undergoing breast surgery and analysed by proton MR spectroscopy at 360 MHZ (8.5 Tesla) and results compared with histopathological analysis. The results of blinded MR analysis were compared with currently employed diagnostic approaches used in assessment of breast lesions. Invasive carcinoma was identified spectroscopically by an increased signal at 3.25 parts per million (ppm) attributable to choline-containing metabolites. Discrimination between invasive carcinoma (n=82) and normal or benign (n=106) or carcinoma *in situ* (n=17) was made based on the intensity of the 3.25 ppm resonance standardised to the resonance intensity at 3.05 ppm ($p<0.0001$, Mann-Whitney U test). An MRS ratio of <1.7 was recorded for 102 out of 106 normal or benign lesions. All carcinoma-*in-situ* samples with comedonecrosis or a microinvasive component (n=6) were ranked by MRS with invasive carcinoma while those with *in-situ* disease alone ranked with benign (n=11). The sensitivity and specificity of MRS on FNB for benign versus invasive breast cancer was 95% and 96% respectively.

BODY

PURPOSE

1. To assess the sensitivity and specificity of ^{1}H MRS (*ex vivo*) in the detection of neoplasia in breast, based on altered cellular chemistry.

2. Correlate alterations to MRS detectable cellular chemistry associated with breast tumour development and progression with established clinicopathological criteria.

3. Ascertain MRS markers which correlate with known clinical, epidemiological and genetic risk factors to be used to identify women at-risk of developing breast cancer.

HYPOTHESES

Proton magnetic resonance spectroscopy can:-

1. Identify altered cellular chemistry in breast tissue independent of method of biopsy eg. open biopsy or fine needle biopsy.

2. Distinguish invasive cancer from normal breast tissue.

3. Distinguish between breast cancers of different type (e.g. lobular or ductal) and grade.

4. Distinguish between progressive and non progressive `carcinoma *in situ*'.

5. Distinguish between `normal' breast tissue and `normal' breast tissue from women with differing risk factors for breast cancer (including age, pre- and post menopausal status, cyclic hormonal effects, genetic predisposition and family history).

6. Identify MRS markers of potential for malignancy in morphologically normal tissue from high risk patients.

7. Independent of histopathology, predict tumour behaviour and clinical outcome (e.g. response to therapy, patterns of metastases) and thereby be a significant predictor of patient survival.

EXPECTED RESULTS

- Expertise in optimal handling and methodological protocols for assessment of breast tissue. **(Completed)**

- An MRS data bank on a full spectrum of breast tissue from normal (i.e. benign) to high grade malignancy. This will allow discrimination of new subsets of cancer-bearing patients for streaming into different management regimes with attendant improved psychosocial, cost efficiency and clinical outcomes. **(Underway)**

- Identification of new cellular chemistry parameters of tumour development in the breast. **(Underway)**

- Definition of MRS markers which correlate with a predisposition for breast cancer development. **(Underway)**

- Statistical verification of MR diagnostic criteria. **(Underway)**

RESEARCH PLAN AND PROGRESS TO-DATE

A. SAMPLE PREPARATION

Objective 1.
Optimise specimen handling and MR data acquisition protocols.

Rationale: Breast tissue contains substantial levels of fat which mask the diagnostic markers of tumour development and progression. Removal of as much of this fat component as possible from the biopsy prior to MRS analysis is both achievable and necessary.

Results: **MRS handling criteria are established and global specimen handling protocols determined to ensure adequate histological correlation of MRS data on the test samples (see appendix II).**

Objective 2.
Ascertain if the same MR criteria can be obtained on fine needle biopsy or aspirate.

Rationale: If the same MR information can be obtained from a fine needle aspirate or needle core biopsy, open surgery may not be a prerequisite for determining the diagnostic parameters.

Results: **A previous study of MRS analysis on FNB of thyroid had established that as few as 10^6 cells were required to obtain one-dimensional MR spectroscopic data with adequate signal-to-noise in less than 15 minutes (256 accumulations) (39). To achieve an adequate breast sample, FNB was performed using multiple (typically 6) aspirated passes (23 gauge needle) either through the resected lesion *ex vivo* (n=129) or *in vivo* (n=89) after lesion identification during open biopsy. These techniques could be guaranteed to provide sufficient cells from the lesion and in addition, allowed the aspiration site to be identified at excision. Tissue from the aspiration site ($3mm^3$) was collected for correlative histopathology. Prior to the ^1H MRS experiment, each FNB specimen was thawed and transferred directly to a 5 mm MRS tube. The volume was adjusted to 300µl with PBS/D_2O where necessary. The sample tube was fitted with a capillary insert containing 60 µl para-aminobenzoic acid (10 mM in PBS/D_2O) as an external standard.**

B. CORRELATION WITH HISTOLOGICAL CRITERIA

Objective 3.
Identify MRS criteria to facilitate distinction between resected carcinoma and normal tissue.

Rationale: The primary data base will be obtained from this section of the study. The 1D and 2D MR data are likely to contain the majority of resonances that need to be assigned to chemical species, biological criteria and clinicopathological criteria.

Results: **All pathological and MRS analyses were undertaken in a blinded study. Correlation of MRS data with clinicopathological criteria were made after all reports were filed. 218 FNB and tissue specimens for MRS analysis and correlative histopathology respectively were obtained from 191 consecutive patients undergoing diagnostic biopsy or definitive treatment (lumpectomy, quadrantectomy or mastectomy) for histologically proven invasive breast cancer. Indications for surgery included mammographically detected impalpable lesions as well as palpable mass lesions which were suspicious by mammography, FNA cytology and/or clinical examination. The age**

range of patients was 20 to 81 years (mean ± SD, 52 ± 14). Where mastectomy for invasive carcinoma was performed, control specimens of macroscopically uninvolved breast tissue (which was later confirmed histologically) were obtained in the same patient (n=27). The MR experimental methods, data processing and analysis, peak assignment procedures and histopathological assessment protocols are described in detail in Appendix II.

Invasive carcinoma was identified by resonances at 3.25 ppm attributable to choline-containing metabolites (Appendix II, Figure 2). A discrimination between invasive carcinoma (n=82) and normal or benign tissue (n=106) was made based on the intensity of the 3.25 ppm resonance standardised to the resonance intensity at 3.05 ppm containing contributions from creatine, phosphocreatine and lysine ($p<0.0001$, Mann-Whitney U test)(Appendix II, Figure 3). A receiver operating characteristic (ROC) curve using this intensity ratio is shown in Appendix I, Figure 4.

Of 106 benign samples, 102 gave a 3.25/3.05 ppm intensity ratio of less than 1.7. Four false positive results were obtained from 3 palpable fibroadenomas, none of which had FNA cytology performed, and 1 lesion comprising moderate ductal hyperplasia. The diagnoses were based on correlative histopathology. FNB from 4 of 82 carcinoma had a ratio of < 1.7. Correlative histopathology from the aspiration site showed that one sample had only benign fibrocystic changes in this region. The other three samples were confirmed as invasive carcinoma but with a marked inflammatory cell infiltrate.

Clinical Correlations: All cases presenting as mammographically suspicious (n=56), mammographically negative (n=23), or non-diagnostic (n=14) or atypical/suspicious (n=25) FNA cytology, were accurately categorised by MRS (as benign or malignant) as confirmed by histopathology on tissue excised from the aspiration site. MRS on F.B. correlated 96% with the final histological diagnosis of benign lesions (Appendix II, Table 2) and yet, biopsy was performed because of clinical (34%) and/or mammographic features (45%) and/or FNA cytology (31%). MRS on F.B. correlated with a malignant histological diagnosis in 95% of cases (Appendix II, Table 2). While combined triple assessment indicated biopsy for all the malignant cases studied, no single pre-operative modality was an improvement on MRS in identifying malignancy (physical examination 84%, mammography 82%, FNA cytology 92%).

Objective 4.
Establish whether MRS can identify differences between variants of breast cancer (e.g. lobular and ductal carcinoma) which reflect altered biological behaviour (e.g. likelihood of response to treatment or location of secondary tumours).

Rationale: Lobular and ductal carcinoma have quite different patient outcome. Ductal carcinoma will usually metastasise to the liver, brain, bone, lungs etc.. whereas lobular carcinoma frequently metastasises to unusual sites. If spectral differences exist between these two types of breast carcinoma and can be assigned to specific biological criteria eg. altered cell surface glycosylation which could reflect altered immunosuppression (50), ability to lodge at different sites (51-53), or adhesion properties (54), the patient outcome could be rationalised at an early stage in the diagnosis and treatment altered accordingly. The reasons for variable survival of women with established metastatic disease may be manifest in the cellular chemistry of the carcinoma cells.

Experimental: **Underway**

Objective 5.
Correlate MRS properties with the established pathological characteristics of breast epithelial hyperplasia and neoplasia.

Rationale: It is important to identify which MRS characteristics are consistent with established pathological criteria. MRS will be used, as an adjunct to current histopathology, with each method contributing to a more thorough and refined diagnosis and patient management.

Experimental: **Underway**

C. CAN MRS REDEFINE THE PRECURSOR STATES OF BREAST NEOPLASIA?

Objective 6.
Compare simple hyperplasias, atypical hyperplasias and malignant tumours. Does MRS identify sub groups or categorise these histological states differently?

Rationale: The strength of the MR method as demonstrated in its application to other organs, was the identification of alterations to cellular chemistry which were not morphologically manifest, or which discriminated between subsets with overlapping or identical histological appearances (e.g. follicular adenoma and carcinoma of thyroid).

Experimental: Underway

Objective 7.
Assess resected tissue containing morphological carcinoma *in situ* to determine if the MR method can separate those, if any, which contain cells committed to invasion from those that cannot progress at that time.

Rationale: Diagnosis of carcinoma *in situ* (CIS) of the human cervix relies on the pathologist confirming that the cells (which are morphologically indistinguishable from invasive cancer) have not invaded. MRS has clearly shown that CIS of the cervix does not have the same cellular chemistry as those cell which are invasive. Does CIS of the breast have a different cellular chemistry from invasive cells? Can the distinction be made between a CIS which has cellular capability to invade from those which have yet to develop fully invasive properties?

Results: **Specimens reported as ductal carcinoma-*in-situ* (DCIS) by routine hospital histopathology were obtained and assessed by MRS. Samples containing only DCIS (10 high grade, 1 low grade) all gave MR ratios $\leq$ 1.7 indicative of a low choline to creatine/lysine ratio similar to that obtained for benign lesions. Ductal cells had breached the basement membrane (< 1mm) in one or more focus of the entire DCIS specimen in the 4 samples denoted 'microinvasion'. This group as well as 2 samples of high grade DCIS with extensive comedonecrosis gave ratios > 1.7 similar to that obtained for malignant lesions.**

D. CAN MRS IDENTIFY CHEMICAL PROFILES ASSOCIATED WITH A PREDISPOSITION TO BREAST CANCER?

Objective 8.
Study morphologically normal tissue from women with differing risk factors for development of breast cancer.

Rationale: It is most likely that alterations to cellular chemistry are able to be documented in the above categories. Are there specific changes which can be identified in at-risk patients?

The effect of :-

age,
pre vs post menopausal status,
cyclic hormonal effects,
genetic predisposition,
family history,

on the spectral profile will be considered in a retrospective study in association with the breast data bank registries.

Experimental: **Underway.**

E. LONGITUDINAL STUDY

Objective 9.
Statistical analysis to correlate MRS data with clinicopathological, epidemiological and genetic data.

Rationale: By examining tissue specimens by MRS, it may be possible to predict the precise progression of breast cancer in individual patients more accurately than histopathology.

Experimental: **Underway**

METHODS *(As in original submission)*

THE SELECTION OF PATIENTS

Biopsy specimens are to be obtained at the time of surgery on 500 patients undergoing surgery to excise benign and malignant lesions by participating surgeons. The indications for surgery include:

1. Mammographically detected impalpable lesions where malignancy cannot be excluded. Stereotactic fine needle aspiration biopsy will be the indicator for surgery.

   In this subgroup approximately 50% will have small invasive cancers, 30% of patients can be expected to have benign lesions such as sclerosing adenosis whilst the remainder will have premalignant conditions like atypical hyperplasia through to carcinoma *in situ*.

2. Excisions of mass lesions which have been proven by mammography, fine needle biopsy, cytology and clinical examination to be malignant where breast conservation can be performed. This is generally for patients with smaller tumours but is dependent upon other factors such as breast size.

The attending surgeon would undertake to provide this follow-up through out this period with regular reports to a central registry office. Initially, patients will be recruited to the study consecutively. However, it is anticipated that, as the study progresses, selection criteria may be introduced to enhance the data base of specific subgroups of patients.

THE PARTICIPATING SURGEONS

THE ROYAL ADELAIDE HOSPITAL

South Australian clinical material in the first instance will be obtained from the patients treated by one surgeon (PLM) from the Breast Endocrine and Surgical Oncology Unit at the Royal Adelaide Hospital. This unit treats 200 primary or new breast cancers per year and provides a dedicated service to a 900 bed teaching hospital on the campus of the University of Adelaide. All patients are treated according to protocol and management is reviewed by a multidisciplinary team prior to treatment. Patients are regularly entered into Australian, New Zealand and international trials where appropriate. The routine pathology service is provided by pathologists from the Institute of Medical and Veterinary Science who also provide a dedicated service and attend the multidisciplinary meetings.

The unit is undertaking research in the following fields:

1. Endocrine responses to oestrogen and progesterone in breast cancer with particular reference to insulin-like growth factor binding proteins.
2. The immunological response associated with human breast cancer with reference to tumour infiltrating lymphocytes (TILs) boosted by lymphocyte growth factors.
3. The evaluation of magnetic resonance imaging of the breast in two subgroups of patients:
   a) young women with dense parenchymal breast tissue
   b) for the evaluation and accurate assessment of women with T3 breast cancers.
4. Dynamic doppler studies to evaluate vascular function in breast reconstruction using autologous tissues.
5. Angiogenesis in breast tumours determined by dynamic doppler.
6. A comparative study of carbon track localisation hook wire for mammographically detected impalpable breast lesions.
7. On-going evaluation of the following tumour markers: **a)** vimentin, **b)** cERB2, **c)** ER, **d)** PR, **e)** CA125, 153.
8. Open label study of high dose chemotherapy in patients with breast cancer using autologous

peripheral blood, stem cells and G-CSF support.

All patients treated by the unit are reviewed at regular intervals according to protocols and information provided to the Cancer Registry.

The unit works in close association with the South Australian Breast X-ray Service which screens 30,00 women per year between the ages of 40 - 69 years. From 1994 the number of women screened will increase to 45,000 per annum. This service provides a constant supply of small tumours as 70% of lesions detected by the clinic are less than 2 cm in size. This service is closely audited and offers the service to the point of diagnosis with particular emphasis upon stereotactic fine needle aspiration biopsy and ultrasound guided needle biopsy. The program is independently audited.

This service can provide biological material ranging through benign, premalignant, ductal carcinoma *in situ* and carcinoma.

WESTMEAD HOSPITAL

The Breast Surgery Unit at Westmead Hospital is multidisciplinary involving all aspects of breast cancer patient care, with a major commitment to the management of patients with breast cancer irrespective of the stage of the disease at presentation. Approximately 150 patients with breast cancer are treated each year within the unit, with approximately 10% having advanced breast cancer and with an increasing number having *in situ* disease as the number of patients accessed through the Screening Unit increases.

The Unit is undertaking research in the following fields:

1. Since 1979 patients with operable breast cancer have had the option of a breast conservation approach involving `lumpectomy', or `quadrantectomy' with clear surgical margins, axillary clearance, and radiotherapy to the breast. A particular form of axillary clearance has been developed with improvements in the way the procedure can be taught to trainees and a video tape is in production and will be shown next year at surgical meetings of the Royal Australasian College of Surgeons.
2. Approximately 50 - 55% of the patients with operable breast cancer currently achieve breast conservation. An initial cohort of approximately 130 patients undergoing a breast preservation procedure have been followed and the results have been reported at 5 and 10 years. Patients needing mastectomy have been treated with total mastectomy and axillary clearance again using the axillary clearance technique developed by the unit.
3. A study has recently been completed to determine the value of the cERB2 antigen in predicting recurrence in node negative breast cancer patients followed for more than 5 years.
4. Apart from its own trails, the unit takes part in a number of international studies including the Zoladex trial for node positive breast cancer.
5. Ongoing evaluation of the following markers is being undertaken: **a)** ER, **b)** PR, **c)** ploidy, **d)** epidermal growth factor receptors.

The surgical team works closely with Radiation and Medical Oncologists, with the Breast Screening Unit and with the Cytology and Pathology Departments. Combined clinics are held and a major Breast Cancer Data Base has been established within the Radiation Oncology Department. Reports have been published on the efficacy of cytology prior to breast surgery, on the problems encountered with the procedures involved in breast conservation, and also the results from our `Advanced Protocol Treatment' of advanced breast cancer at presentation. The senior surgeon is also the senior surgeon of the Screening Unit which now has considerable expertise in all aspects of Screening, and is recognised as a training unit for medical and paramedical professionals involved with Breast Screening. Surgical and radiotherapy trainees take part in the clinical program and for the past 12 months a `Breast Fellow' has been appointed and this is a recognised post for the Royal Australasian College of Surgeons for postgraduate training breast surgery for young surgeons who have achieved Fellowship of the Royal Australasian College of Surgeons.

This service can provide biological material ranging through benign, premalignant, ductal carcinoma *in situ* and carcinoma.

CONCORD HOSPITAL

The Breast Endocrine Unit at Concord Hospital was established in 1986. It is composed of four dedicated surgeons working from a forty bed unit. It is closely associated with the University of Sydney and is involved in student teaching, registrar training and research.

Approximately 100 cases are seen per annum. The data collection is made on a standard protocol and processed using computer programs.

The unit is associated with the breast screening clinics of Central and Western Sydney. The principals attend these clinics and patients are then referred for treatment at Concord Hospital.

Multi-disciplinary meetings involving pathologists, radiologists and surgeons are held on a regular basis and a routine monthly review of all cases is undertaken. Standard protocols for breast treatment have been instituted and are followed by the treating surgeons.

Papers have been published on the accuracy of fine needle aspiration cytology in breast cancer patients, the incidence of mammography negative breast cancers seen through the clinic and the incidence of multi focal breast cancer.

This service can provide fewer specimens than the larger two centres but a strong collaboration between the MR Unit and surgeons at Concord Hospital is well established.

MAGNETIC RESONANCE SPECTROSCOPY

Sample Handling Tissues obtained at surgery will be placed into a sterile tube containing pre-cooled (4 C) phosphate buffered saline in D_2O (PBS/D_2O) immediately after excision. Alternatively, biopsies will be snap frozen in liquid nitrogen, transported to the laboratory and stored at -70 C until ready for examination. Samples to be examined by MRS will be gently thawed, washed in PBS/D_2O (5 x 1 ml) and placed in a 5 mm MR tube containing sufficient PBS/D_2O to cover the biopsy. Placement within the transmitter/receiver coil will be ensured by either resting the biopsy on top of a plug of glass wool (5), or by suspending the biopsy inside an inner capillary tube of 2.5 mm diameter (10,34). In the case of the capillary method, the external volume will be filled with 350 µl of 1 mM p-aminobenzoic acid (PABA) in PBS/D_2O which serves as a chemical shift and concentration reference (34). As in the case of colorectal biopsies (21), prior to the MRS experiment the specimens will have excess adipose tissue and vasculature excised. Alternative methods such as passing the tissue through a metal sieve to trap the fat (see Page 17) will be explored.

F.B.: will be performed using 5 aspirated passes through the specimen with a 21 gauge needle (39). Previous studies (55) have shown that this combination gives the greatest number of cells, both single and in clumps, although it is not the preferred technique if clear cellular detail is required for accurate cytological examination. F.B. are washed with 2 x 1 ml PBS/D_2O. Between washes the samples are centrifuged (1000g for 5 min) and the supernatant discarded. The F.B. are suspended in PBS/D_2O (final volume 125 µl) and then placed on top of a glass wool plug in a 5 mm MRS tube.

Data Collection Data will be collected on a Bruker AM360 wide-bore MR spectrometer equipped with an Aspect 3000 computer and a dedicated 5 mm or 8 mm ^1H probe. Temperature is maintained at 37 C using a Bruker VT1000 temperature regulation unit.

Pulse Sequences All pulse sequences to be used are functional in this laboratory at the time of application. Typical experiments are as follows:-

1D ^1H spectroscopy: 1D spectra (at 360 MHZ) are obtained using a spectral width of 3600 Hz (10 ppm) and 8K data points. A relaxation delay of 2 sec is used during which gated decoupling (15 dB below 0.2 W) is applied for the final 1 sec to reduce the residual water signal (56). 128 transients are averaged.

CPMG: One-dimensional T_2- filtered experiments are performed using the Carr-Purcell-Meiboom-Gill pulse sequence (90_x-(-180_y- -)$_n$-acquire) with an interpulse delay of = 1 msec and a 2 sec relaxation delay between acquisitions (48). Gated presaturation is used in the final 1 sec before data acquisition. 128 transients are collected using a spectral width of 10 ppm. T_2- filtered 1D experiments on biopsy specimens is performed using selected values of n. Typical values of n2 = 16, 480, 720, 960 ms.

2D ^{1}H- ^{1}H COSY spectroscopy: Magnitude-mode COSY spectra are performed using a standard two pulse sequence with the two pulses separated by an incremented delay (57). The sweep width in the t_2 domain is 3000 Hz, and the size in the t_2 domain is 2K data points. The initial delay between the 2 pulses is 1 msec with an increment time of 334 sec. There is a relaxation delay of 1 sec before each accumulation during which the water resonance is presaturated using a CW irradiation power of 30 dB below 0.2 W. The number of time domain points collected in t_1 (free induction decays, FIDs) is subject to the viability limits of the sample. Tissue experiments consist of 180-220 FIDs, each of 32-48 transients (plus 2 dummy scans) over a total experiment time of three to five hours.

T_2-filtered COrrelation SpectroscopY: T_2-filtered correlation spectra are obtained by replacing the first pulse of a standard COSY pulse sequence with a CPMG sequence (48). Typical parameters are described above with n2 equal to 500 - 750 msec. The sequence removes crosspeaks arising from short T_2 species, like lipid, leaving only resonances from more mobile metabolites. The pulse sequence is useful in resonance assignment in specimens, like breast, which contain large quantities of lipid that can be reduced or removed using this technique.

Processing of MRS Data Data will be processed on a Bruker X32 (UNIX) Data Station. The complexities of processing FIDs from biological material containing a wide range of T_2 relaxation values (0.3 - 1.5 s) has been addressed in this laboratory (17). The processing parameters vary according to the MR visible chemicals of each tissue type.

1D spectroscopy: One dimensional spectra (1D and CPMG) are routinely processed using a line broadening of 3 Hz applied before Fourier transformation. Data are phase corrected using zero and first order phase correction and baselines are corrected using a fourth order polynomial baseline correction routine. Linewidths or ratios of peak heights are measured in 1D spectra.

2D spectroscopy: COSY data matrices undergo zero filling to 1K in t_1, Fourier transformation and magnitude calculation ((Real^2 + Imaginary^2)) to give 1024 x 1024 real data points for each COSY spectrum. Sine-bell window functions are uniformly applied in the t_1 domain and Lorentzian-Gaussian window functions of varying width and maximum positions are applied in the t_2 domain prior to Fourier transformation as previously described (17). Crosspeak volumes and subsequent ratios to external or internal reference peaks will be measured as previously described (2,7,8,58).

Referencing peak height integrals, or peak heights, to those of an external standard with a constant concentration and T_2 relaxation value allows a semi quantitative concentration measurement. However, differences in crosspeak volumes can arise from either concentration changes or from changes in T_2 relaxation of the species. These two effects cannot be separated, but an increase in crosspeak volume will reflect either a chemical change or a reorganisation of the compound within the cell, and may still have diagnostic relevance.

MATHEMATICAL ANALYSIS OF MR DATA FROM THE UNIX

Unprocessed MRS data will be transferred electronically via internet to the Silicon Graphics server at the Institute for Biodiagnostics, Winnipeg, Canada for a thorough mathematical analysis. The MR spectra will be prepared for analysis by means of the proprietary software ALLFIT (National Research Council of Canada), which provides appropriate baselines and integrals for each distinguishable peak. The spectra will then be preprocessed by formation of a correlation matrix to avoid problems due to widely disparate variate ranges. Principal component analysis will then be applied to the spectral regions of interest, in order to reduce the number of variable in the computation. One half the available data will be used as a training set, and the remaining half will be classified blindly. To make all analyses robust,

the training set data will be analysed by the leave-one-out method *i.e.* train on k-1 of the k samples, classify the sample that was excluded from the training set, and repeat this k times, once for each sample.

Classification will be performed by a variety of methods, alone and in combination. The principal methods are linear and quadratic discriminant analysis (45), neural nets of various types (44), and genetic programming (46). Consensus analysis will be performed within each method using different regions of the spectrum and with combinations of methods, to increase accuracy of classification. Fuzzy logic methods will be applied where appropriate.

HISTOPATHOLOGY

Every MR sample will be assessed by one of two pathologists.

Expertise of the Pathologists The two pathologists participating in this study are Senior Hospital Consultants, each with over 25 years experience. Professor P. Russell is an acknowledged authority (59) and Dr J. Phillips is well recognised internationally in the field of aspiration cytology. Each pathologist has indicated that he/she is willing and able to make the necessary contribution in time to ensure the success of this project.

Rationale for the Extent of Histological Assessment

1. Primary correlation is obtained by comparing the MRS result with standard hospital histopathological diagnosis. This involves no additional time spent by the pathologist. Long-term storage of fixed tissue for later re-examination will be required.
2. The MRS sample is always examined **initially** by six "step-sections" 7μm sections (taken at 350 μm intervals) at x 40 magnification. The intervening sections are mounted, stained and stored for future assessment if required. Tissue preservation, cellular content and presence of potentially confounding factors such as fat and inflammatory cells are reported in addition to the diagnosis. This involves an additional 5-6 minutes of pathologist time per specimen and is undertaken without reference to clinical or MRS data (in both MRS specimens and remainder of surgical specimen).
3. Where disagreement exists between MRS diagnosis and histological diagnosis, the MRS sample will be step-serially sectioned every 7 μm (refer to **2**) and examined by the pathologist in a blind study. To avoid bias other specimens will be included at the same time.

Tissue Preparation

Routine: Tissue is fixed in 10% buffered formalin or FAA (formalin:acetic acid:alcohol), paraffin embedded and sectioned, stained with haematoxylin and eosin according to standard protocols.

Serial sectioning: Routine sectioning of paraffin blocks will be at 7 μm. Step serial sectioning of paraffin blocks will be undertaken on selected specimens.

Criteria for establishing firm histological diagnosis of cancer and its precursors in the tissues to be included in this study, will follow established guidelines. In general terms, the various pathological subtypes of cancer will be classified according to the relevant WHO International Histological Tumour Classification and, where difficult diagnostic problems are encountered (particularly for borderline malignancies), the guidelines in the following reference text will be used *viz* Page D.L. & Anderson T.J. (1990) Diagnostic Histopathology of the Breast. Churchill Livingstone, United Kingdom.

Quantification of Cell Types This will be performed usually on `step' or `full' serial sections and is only intended to provide an approximate guide to relative proportions of study tissues to background stroma and reactive inflammatory infiltrates etc.

STATISTICAL ANALYSIS

The epidemiologist (O.D.) in Canberra will examine associations statistically between MRS assessment and pathology and clinical variables using comparisons between means, contingency tables and logistic regression. Associations between MRS spectra and patient survival will be assessed by Kaplan - Meier Survival analysis (60) and proportional hazards regression models (61).

CONCLUSIONS

- **This project is proceeding as outlined in the original document and as described above.**
- **Of the three "purposes" listed on page 4 the first two are well underway.**
- **Hypotheses 1 and 2 (page 4) have been shown to be correct. Data collected so far indicate that hypothesis 4 is also correct.**
- **No unforseen difficulties have been encountered and the program is proceeding to schedule.**

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APPENDIX I

ACRONYM /SYMBOL DEFINITION

1D	-	one dimensional
2D	-	two dimensional
^1H	-	proton
12p	-	chromosome 12 p (short) arm
18q	-	chromosome 18 q (long) arm
CA125	-	tumour marker
CA153	-	tumour marker
cERB2	-	tumour marker
CH/CH_2	-	methine to methylene ratio
CH_2/CH_3	-	methylene to methyl ratio
CIN	-	cervical intraepithelial neoplasia
CINI	-	cervical intraepithelial neoplasia - stage I
CINII	-	cervical intraepithelial neoplasia - stage II
CINIII	-	cervical intraepithelial neoplasia - stage III
CIS	-	carcinoma *in situ*
CPMG	-	Carr-Purcell-Meiboom-Gill
COSY	-	COrrelated SpectroscopY
CW	-	continuous wave
dB	-	decibel
D_2O	-	deuterium oxide
ER	-	oestrogen receptor
f_1 and f_2	-	frequency in the first and second dimensions of a 2D MR experiment
FAA	-	formalin:acetic acid:alcohol
FID	-	free induction decay
FNB	-	fine needle biopsy
Fuc	-	fucose
GB	-	Gaussian broadening
G-CSF	-	granulocyte colony stimulating factor
H_5-H_6	-	coupling between protons attached to the C_5 and C_6 of carbohydrate moieties

Hz	-	Hertz
K-*ras*	-	oncogene
LB	-	Lorentzian broadening
Le^y	-	$Lewis^y$ (antigen)
MHz	-	mega Hertz
MRI	-	magnetic resonance imaging
MRS	-	magnetic resonance spectroscopy
MR	-	magnetic resonance
NRC	-	National Research Council of Canada
p53	-	tumour supressor gene
PABA	-	p-aminobenzoic acid
PBS	-	phosphate buffered saline
PCA	-	principal component analysis
ppm	-	parts per million (units of chemical shift)
PR	-	progesterone receptor
SFNAB	-	stereotactic fine needle aspiration biopsy
t_1 and t_2	-	first and second time domains in a 2D MR experiment
T1	-	tumour size less than 2 cm
T2	-	tumour size 2 - 5 cm
T3	-	tumour size greater than 5 cm
T_2	-	spin-spin (transverse) relaxation
Thr/Fuc	-	threonine/fucose
TIL	-	tumour infiltrating lymphocytes

FINE NEEDLE BIOPSY OF BENIGN BREAST LESIONS DISTINGUISHED FROM INVASIVE CANCER EX VIVO BY PROTON MAGNETIC RESONANCE SPECTROSCOPY

Wanda B. Mackinnon, PhD, Peter Barry, MB BS, +Peter Malycha, FRACS, †David Gillett, MS, FRACS, Peter Russell, MD, FRCPA, Cynthia L. Lean, PhD, Sinead Doran, B.Sc.(Hons), §Bruce Barraclough, FRACS, DDU, ¥Michael Bilous, FRCPA, and Carolyn E. Mountford*, D.Phil.

Institute for Magnetic Resonance Research, University of Sydney, N.S.W. 2006, Australia.

+Department of Surgery, Royal Adelaide Hospital, Adelaide, South Australia 5000

†Department of Surgery, Repatriation General Hospital, Concord, N.S.W. 2137, Australia

§Departments of Surgery and ¥Anatomical Pathology, Westmead Hospital, Sydney, N.S.W. 2145, Australia.

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Running Title: Breast pathology identified on FNB by Proton MRS.

*To whom correspondence should be addressed.

Fax: 612 - 9351-4105 email: caro@medicine.su.oz.au

ABSTRACT

PURPOSE To prospectively determine whether conventional unlocalized proton magnetic resonance spectroscopy (^{1}H MRS) *ex vivo* can distinguish invasive breast cancer from benign lesions based on altered cellular chemistry.

MATERIALS AND METHODS Fine Needle Biopsies (FNB) (n=218) were obtained from patients undergoing breast surgery and analysed by proton MR spectroscopy at 360MHz (8.5 Tesla) and results compared with histopathological analysis. The results of blinded MR analysis were compared with currently employed diagnostic approaches used in assessment of breast lesions.

RESULTS Invasive carcinoma was identified spectroscopically by an increased signal at 3.25 parts per million (ppm) attributable to choline-containing metabolites. Discrimination between invasive carcinoma (n=82) and normal or benign (n=106) or carcinoma *in situ* (n=17) was made based on the intensity of the 3.25 ppm resonance standardised to the resonance intensity at 3.05 ppm ($p<0.0001$, Mann-Whitney U test). An MRS ratio of <1.7 was recorded for 102 out of 106 normal or benign lesions. All carcinoma-*in-situ* samples with comedonecrosis or a microinvasive component (n=6) were ranked by MRS with invasive carcinoma while those with *in-situ* disease alone ranked with benign (n=11). The sensitivity and specificity of MRS on FNB for benign versus invasive breast cancer was 95% and 96% respectively.

CONCLUSION ^{1}H MRS on FNB provides objective diagnostic information which potentially complements conventional pre-operative investigations in women with breast lesions.

Key Words:

nuclear magnetic resonance
breast cancer
cytology
carcinoma *in situ*
fine needle aspiration

INTRODUCTION

In Australia, breast cancer is the leading cause of cancer-related death in women. Its incidence outranks all other cancers in women over age 35 years. In the past decade, the incidence rose by 25% and the life-time risk (from 0-74 years) for white women developing breast cancer is comparable to Western world figures at 1 in 13.

Recent improvement in breast cancer patient outcome is due to earlier diagnosis and more effective management.[1,2] A combination of physical examination, mammography and fine needle aspiration cytology (triple assessment) is to date the most sensitive method for pre-operative diagnosis of clinical and radiographically detected breast lesions. While triple assessment has a high probability of detecting all malignant lesions, its sub-optimal specificity results in diagnostic uncertainty requiring open biopsy to exclude malignancy in many women.

Physical examination has limitations due to individual variation in breast consistency, the site and size of the lesion (less than 1 cm is usually impalpable), and the presence of a diffuse versus discrete tumour. Screening mammography guidelines ensure that approximately one benign lesion is biopsied for every malignant lesion detected.[3] Even so, 10 - 40% of palpable breast cancers are missed on mammography alone, especially in women under 50 years of age in whom radiographically dense breast tissue may obscure changes associated with malignancy.[4-7] Fine Needle Aspiration cytology (FNA) has a sampling error rate in the range of 1 to 15%.[8] partly explaining its varied sensitivity. While the complete sensitivity of FNA is 81-97%,[9] this includes atypical and suspicious diagnoses which typically lead to the histological confirmation of cancer by open biopsy in 50 to 80% of cases.[10]

A technology which monitors cellular chemistry which correlates with different cell behaviour could offer both independent and objective assessment of breast tissue. The potential then exists to identify a predisposition towards or early features of breast cancer and thus offer interventions aimed at reducing tumour development. Proton Magnetic Resonance Spectroscopy (MRS) is one diagnostic

modality which has been successfully applied to monitor tumour development and progression in other organs.[11-17] Proton MRS can distinguish pre-invasive from invasive cancer of the uterine cervix with a sensitivity and specificity of 98% and 94% respectively[12]. The technique also distinguishes genuinely benign from malignant follicular lesions in human thyroid[17] and discriminates degrees of loss of cellular differentiation in ovarian tumours.[15]

Preliminary investigations of excised breast tissue by unlocalized one- and two-dimensional proton MRS detected increased levels of glycerophosphocholine and phosphocholine in invasive breast carcinoma compared with benign fibroadenomas.[18] Specimens of benign fibrocystic disease were characterised by an absence of resonances from choline, amino acids and other metabolites. However, a major difficulty was experienced in obtaining adequate spectral resolution from excised breast due to the high adipose content of the tissue. The intense broad MR resonances from this fat often masked other diagnostic resonances in the one-dimensional spectrum. This problem is largely overcome by applying a T_2 filter (Carr-Purcell-Meiboom-Gill sequence) during data collection[19] and/or with post-acquisitional data processing.[20] A simpler remedy was to optimise specimen collection. Collection procedures were found to substantially affect the amounts of exogenous fat sampled. Fine needle aspiration biopsy methods developed for thyroid sampling[16] provided cellular material adequate for assessment by MRS with reduced fat levels compared with excised tissue.[21]

Our objectives for this study were 1) to assess the sensitivity and specificity of ^1H MRS for delineating invasive breast cancer *ex vivo* on FNB, 2) to determine whether carcinoma *in-situ* could be distinguished from invasive carcinoma and 3) to compare diagnosis by MRS on FNB with traditional pre-operative investigations such as physical examination, mammography and FNA cytology.

MATERIALS AND METHODS

All pathological and MRS analyses were undertaken in a blinded study. Correlation of MRS data with clinicopathological criteria were made after all reports were filed.

Patients

218 FNB and tissue specimens for MRS analysis and correlative histopathology respectively were obtained from 191 consecutive patients undergoing diagnostic biopsy or definitive treatment (lumpectomy, quadrantectomy or mastectomy) for histologically proven invasive breast cancer. Indications for surgery included mammographically detected impalpable lesions as well as palpable mass lesions which were suspicious by mammography, FNA cytology and/or clinical examination (see Fig.1). The age range of patients was 20 to 81 years (mean $\pm$ SD, 52 $\pm$ 14). Where mastectomy for invasive carcinoma was performed, control specimens of macroscopically uninvolved breast tissue (which was later confirmed histologically) were obtained in the same patient (n=27).

Specimen collection

A previous study of MRS analysis on FNB of thyroid had established that as few as 10^6 cells were required to obtain one-dimensional MR spectroscopic data with adequate signal-to-noise in less than 15 minutes (256 accumulations).[16] To achieve an adequate breast sample, FNB was performed using multiple (typically 6) aspirated passes (23 gauge needle) either through the resected lesion *ex vivo* (n=129) or *in vivo* (n=89) after lesion identification during open biopsy. These techniques could be guaranteed to provide cells from the lesion and in addition, allowed the aspiration site to be identified at excision. Tissue from the aspiration site ($3mm^3$) was collected for correlative histopathology.

Specimen handling

Cells or tissue were placed in polypropylene vials containing 300 µl phosphate buffered saline (PBS) in D_2O. All specimens were immediately

immersed in liquid nitrogen and stored at -70°C for up to 6 weeks until MRS analysis.

Preparation of specimens for proton MRS

Prior to the ^1H MRS experiment, each FNB specimen was thawed and transferred directly to a 5 mm MRS tube. The volume was adjusted to 300µl with PBS/D_2O where necessary. The sample tube was fitted with a capillary insert containing 60 µl para-aminobenzoic acid (10 mM in PBS/D_2O) as an external standard.

Proton magnetic resonance spectroscopy

^1H MRS assessment of all specimens was performed without knowledge of the final histological diagnosis.

Data acquisition: MRS experiments were carried out on a Bruker AM-360 wide-bore spectrometer (operating at 360 MHz or 8.5 Tesla) equipped with an Aspect 3000 computer and a standard 5 mm dedicated proton probehead. The sample was spinning at 20 Hz and the temperature maintained at 37°C. Residual water signal was suppressed by selective gated irradiation using low power (15 dB below 0.2 W). The chemical shifts of resonances were referenced to aqueous sodium 3-(trimethylsilyl)-propanesulphonate (TSPS) at 0.00 ppm. One-dimensional spectra were acquired as previously described[18] over a sweep width of 3597 Hz (10.0 ppm) using a ninety degree pulse, 8192 data points, 256 accumulations, an acquisition time of 1.14 seconds and a relaxation delay of 2.00 seconds, resulting in a pulse repetition time of 3.14 seconds.

Data processing: Data processing for each specimen was undertaken independently by two operators without access to the histopathology reports, one using the spectrometer's Aspect 3000 computer and the other using a Bruker X32 (UNIX) data station. A line broadening of 3.0 Hz was applied to the data prior to Fourier transformation. Data were phase corrected using zero and first order phase correction. Baselines were corrected using a fourth order polynomial baseline correction routine.

Data analysis: The ratio of the peak height intensities of spectral resonances at 3.25 and 3.05 ppm ("MRS Ratio") was calculated by each operator and the two results for each specimen averaged. This ratio was used to generate Figure 3. Data was rejected for inadequate signal to noise (n=11) if the peak intensity of the methylene (-CH_2-) resonance at 1.3 ppm in the FNB spectrum was less than 40% of the intensities of those generated by the external standard or if the ratio of the -CH_2- signal intensity to noise intensity was less than 25.

Histopathology

The diagnostic correlation was obtained by comparing the MRS Ratio with the post-operative hospital pathology report provided for each patient. The final histological diagnoses in the study group are shown in Table 1.

Without reference to clinical or MRS data, cytological analysis of the aspirate after MRS analysis was attempted. However, cellular detail was compromised by autolytic changes and this approach was not pursued. In order, therefore, to verify FNB sampling accuracy, separate histopathological assessment by a single pathologist (PR) was obtained from tissue removed from the aspiration site of the MRS sample (see *Specimen Collection).* These tissue specimens were thawed, fixed in FAA (formalin/acetic acid/alcohol), paraffin embedded, sectioned at 7 μm, stained with haematoxylin and eosin according to standard protocols and reviewed by the pathologist under the light microscope without access to the clinical or MRS data. Tissue preservation, abundance of epithelial cells relative to stroma and presence of potentially confounding factors such as fat and inflammatory cells were reported in addition to the principal diagnosis.

Other clinical correlations

Comparisons were made to other pre-operative diagnostic investigations including physical examination, mammography and FNA cytology (separate specimen to MRS).

RESULTS

Sample collection

Fine needle aspiration successfully overcame the problem of high levels of MR measurable fat inherent in the analysis of solid breast tissue. The resultant improvement in spectral resolution due to reduction of exogenous fat was confirmed by histopathological analysis (data not shown).

For 207 of the 218 FNB specimens obtained, sufficient cellular material was collected for adequate MR assessment under the chosen experimental conditions. Eleven were discarded for technical reasons (see Methods, *Data analysis*).

Benign lesions versus invasive carcinoma

Invasive carcinoma was identified by resonances at 3.25 ppm attributable to choline-containing metabolites (Figure 2). A discrimination between invasive carcinoma (n=82) and normal or benign tissue (n=106) was made based on the intensity of the 3.25 ppm resonance standardised to the resonance intensity at 3.05 ppm containing contributions from creatine, phosphocreatine and lysine ($p<0.0001$, Mann-Whitney U test) (see Figure 3). A receiver operating characteristic (ROC) curve using this intensity ratio is shown in Figure 4.

Of 106 benign samples, 102 gave a 3.25/3.05 ppm intensity ratio of less than 1.7. Four false positive results were obtained from 3 palpable fibroadenomas, none of which had FNA cytology performed, and 1 lesion comprising moderate ductal hyperplasia. The diagnoses were based on correlative histopathology.

FNB from 4 of 82 carcinoma had a ratio of < 1.7. Correlative histopathology from the aspiration site showed that one sample had only benign fibrocystic changes in this region. The other three samples were confirmed as invasive carcinoma but with a marked inflammatory cell infiltrate.

Two cases of Phylloides tumour were studied. One was multifocal and gave a ratio >1.7, and the second specimen from a single mass gave a ratio of 1.6. Phylloides tumours are classified as a fibroepithelial proliferation with variable biological activity. Their behaviour ranges from benign through a propensity to local

recurrence to blood-borne metastasis. Both phylloides tumours were excluded from all statistical analysis.

Carcinoma in-situ

All samples reported as ductal carcinoma-*in-situ* (DCIS) by routine hospital histopathology are shown in Figure 5. These were grouped according to the correlative histopathology findings. No *in-situ* disease was detected in the correlative histopathology of the 6 samples denoted 'benign'. Ductal cells had breached the basement membrane (< 1mm) in one or more focus of the entire DCIS specimen in the 4 samples denoted 'microinvasion'. This group as well as 2 samples of high grade DCIS with extensive comedonecrosis gave ratios > 1.7. Samples containing only DCIS (10 high grade, 1 low grade) all gave ratios ≤ 1.7 indicative of a low choline to creatine/lysine ratio similar to that obtained for benign lesions.

Clinical Correlations

All cases presenting as mammographically suspicious (n=56), mammographically negative (n=23), or non-diagnostic (n=14) or atypical/suspicious (n=25) FNA cytology, were accurately categorised by MRS (as benign or malignant) as confirmed by histopathology on tissue excised from the aspiration site.

MRS on FNB correlated 96% with the final histological diagnosis of benign lesions (Table 2) and yet, biopsy was performed because of clinical (34%) and/or mammographic features (45%) and/or FNA cytology (31%). MRS on FNB correlated with a malignant histological diagnosis in 95% of cases (Table 2). While combined triple assessment indicated biopsy for all the malignant cases studied, no single pre-operative modality was an improvement on MRS in identifying malignancy (physical examination 84%, mammography 82%, FNA cytology 92%).

DISCUSSION

MRS on FNB when compared with the histopathological diagnosis has a sensitivity and specificity for the differentiation of invasive carcinoma from benign breast lesions of 95% and 96% respectively, based on the 1D peak intensity ratio of 3.25/3.05 ppm. The increase in ratio observed for carcinoma tissues is most likely due to elevated choline metabolite levels in malignant compared to benign tissues. This is consistent with a higher rate of cellular replication, specifically with increased phospholipid synthesis and membrane turnover.

While triple assessment followed by open biopsy has an overall sensitivity approaching 100%, this entails a high number of open biopsies for benign disease. This is especially the case when equivocal or suspicious mammographic findings are followed by either atypical or suspicious FNA cytology or benign cytology which is incongruous with clinical or radiological findings. In our study, all atypical or suspicious FNA cytology results on subsequently confirmed benign specimens gave a 'benign' MRS ratio on FNB. While the MRS was based on an *ex vivo* aspirate, MRS performed prior to cytology on in vivo aspirates may well be a valuable adjunct by improving its specificity. Therefore MRS on FNB could reduce the number of biopsies performed on 'benign' lesions and lead to a more conservative approach such as continued observation/surveillance with repeat FNA cytology and MRS. Used as a complementary modality to triple assessment, MRS on FNB may also provide further diagnostic confidence where open biopsy is requested for patient reassurance only.

MRS on FNB clearly distinguished pure DCIS without comedonecrosis or microinvasion (MRS ratio $\leq$ 1.7) from invasive carcinoma. However, even when DCIS specimens contained comedonecrosis or a few foci of microinvasion, MRS ranked the specimen in the invasive category in every case. This may demonstrate the selective sampling of necrotic cells or invasive disease by the MRS FNB, but could also indicate chemical changes occurring in cells progressing from *in-situ* to frankly invasive prior to morphological manifestation. Pure DCIS was not distinguished from benign lesions based on the 1D 3.25/3.05 ppm ratio but more spectral information is available for comparison of the two groups and this is being

investigated. In particular, two-dimensional MRS may identify specific chemical differences characteristic of pure DCIS. Alternatively, multivariate analysis as previously reported for thyroid neoplasms[22], yet to be undertaken, is likely to improve sensitivity and specificity.

Seventy eight out of 82 invasive carcinoma had an MRS ratio of greater than 1.7. Of the 4 false negative specimens, one was found not to have invasive carcinoma present in the sample which was aspirated. The remaining three samples all had a marked inflammatory infiltrate which, if sampled, may have accounted for a ratio of <1.7. For all remaining carcinoma specimens, including those which were mammographically negative or non-diagnostic on FNA cytology, MRS correctly indicated malignancy.

In this study, FNB for MRS was collected intra operatively from the exposed lesion to ensure accurate sampling. If MRS on FNB were to be used as a pre-operative diagnostic modality, a sampling error may be introduced similar to that experienced by FNA cytology reducing the sensitivity and specificity recorded here. For example, in this patient sample, 14 out of 143 cytology reports were non-diagnostic or insufficient. Further studies will directly compare MRS with cytology obtained on the same aspirate at the time of clinical assessment.

The potential clinical use of MRS on FNB is to complement triple assessment procedures thus reducing unnecessary biopsy of benign lesions. Management of patients with breast lesions varies, however, from country to country. In Australia FNB is routinely used as part of the triple assessment, thus introducing MRS as a fourth modality would not alter patient management. In the USA, core biopsy is more often employed. Core biopsies have two potential areas of usefulness, viz. demonstrating an invasive focus within an area of DCIS; histological confirmation of radiologically suspected benign lesions. It is anticipated that MRS on FNB will be able to address both these questions as or more accurately than core biopsy, avoiding the more invasive procedure. A core biopsy potentially contains high levels of fat making it unsuitable for MRS analysis. Thus, in countries such as the USA the choice of biopsy procedure will need to be reassessed and re-introduction of FNB measured against the improved sensitivity and specificity of the MRS

method.

Furthermore, it is likely that the technical obstacles to *in vivo* spectroscopy of the breast be overcome. It has been shown in at least one USA site that the diagnostic chemical information reported herein is available*in vivo*. Considerable research and development is required, however, before such techniques will be in routine clinical use.

MRS on *ex vivo* FNB may a) complement 'triple assessment' and reduce the need for unnecessary biopsy of benign lesions and b) obviate the need for open biopsy prior to definitive therapy of invasive lesions by increasing diagnostic specificity of cytology. Pre-operative diagnosis of breast lesions by proton MRS thereby offers potential benefits in patient management by reducing potential morbidity related to biopsy and allaying anxiety due to equivocal diagnosis.

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FIGURE LEGENDS

FIGURE 1 Flow chart indicating the management and final histopathological diagnosis of patients involved in the study. The total number of patients sampled was 191. Numbers in parentheses indicate the number of patients who had an uncertain cytological diagnosis during the diagnostic work-up. Numbers alongside arrows indicate the total number of patients. Samples from eleven patients were rejected.

[1] includes invasive and *in-situ* carcinoma.

[2] includes lumpectomy and quadrantectomy. Nine of these patients required a completion mastectomy (not shown).

[3] 12 for *in-situ* carcinoma, 27 for invasive carcinoma

FIGURE 2 One dimensional ^{1}H MRS (256 scans) on FNB taken from the same patient. **(A)** Normal uninvolved breast **(B)** Invasive ductal carcinoma. The distinction between normal breast and invasive carcinoma is based on an increase in the N-trimethyl resonance at 3.25 ppm normalised to that of creatine at 3.05 ppm (peaks indicated by arrows). Data was collected as described in **METHODS**.

FIGURE 3 Plot of the ratio of the intensity of resonances at 3.25 and 3.05 ppm measured from MR spectra as shown in Fig. 2 and as described in materials and methods. Unequivocally benign and invasive lesions are compared. Data are grouped on the basis of the final histopathology of tissue specimens taken from the aspiration site.

FIGURE 4 Relative Operating Characteristic curve of the data from Fig. 3 (total n = 188) calculated from the 'MRS Ratio' of all samples. The ratio was ranked and both the sensitivity and specificity were calculated for all values of the ratio.

FIGURE 5 Plot of the ratio of the intensity of resonances at 3.25 and 3.05 ppm measured from samples reported as ductal carcinoma-*in-situ* on post-operative pathology. Data are grouped on the basis of the final correlative histopathology.

TABLE 1: Summary of Histological Subtypes of FNB samples.

Benign	
Fibrocystic Changes	68
Fibroadenoma	15
Ductal Hyperplasia (mild / florid)	9
Fat Necrosis	4
Sclerosing Adenosis	2
Radial Scar	2
Atypical Ductal Hyperplasia	2
Duct Ectasia	2
Miscellaneous	2
TOTAL	**106**
Phyllodes Tumour	2
Ductal Carcinoma *in-situ*	
DCIS - high grade	10
DCIS - low grade	1
DCIS - comedonecrosis	2
DCIS + microinvasion	4
TOTAL	**17**
Invasive Carcinoma	
Ducal Carcinoma - No Special Type	66
Ductal Carcinoma (NST), EIC positive	7
Lobular Carcinoma	5
Tubular Carcinoma	4
TOTAL	**82**

DCIS = Ductal Carcinoma *in situ*, NST = No Special Type, EIC = Extensive Intraductal Component. Histopathology according to dominant findings on correlative histopathology samples.

TABLE 2: TEST RESULT PROBABILITIES FOR DIAGNOSIS BY MRS ON FNB COMPARED WITH HISTOLOGICAL DIAGNOSIS ON TISSUE.

	Invasive Carcinoma	**Normal/Benign**	
MRS (FNB) Positive	78	4	PPV 95%
Ratio ≥ 1.7	(TP)	(FP)	(78/82)
MRS (FNB) Negative	4	102	NPV 96%
Ratio < 1.7	(FN)	(TN)	(102/106)
	Sensitivity 95%	**Specificity 96%**	**Total=188**
	(78/82)	(102/106)	

TP = True positive; FN = False negative; FP = False positive; TN = True negative; PPV = Positive predictive value; NPV = Negative predictive value; Sensitivity or true-positive rate = frequency of positive test result (ie. MRS ratio ≥ 1.7) in those with malignant disease; Specificity or true-negative rate = frequency of negative test result (MRS ratio < 1.7) in those without malignant disease as judged by histopathology of breast tissue excised from the aspiration site.

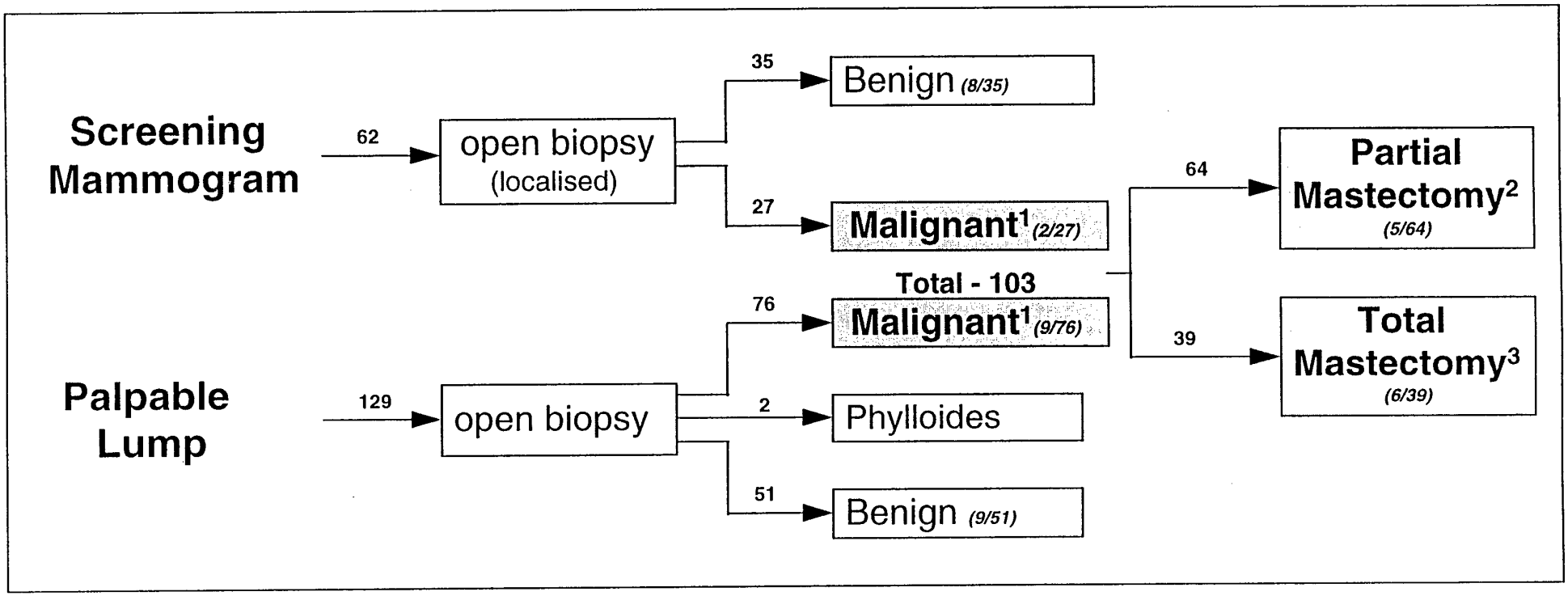
Screening Mammogram
62
open biopsy (localised)
35
Benign (8/35)
27
Malignant1 (2/27)
Palpable Lump
129
open biopsy
76
Malignant1 (9/76)
2
Phylloides
51
Benign (9/51)
Total - 103
64
Partial Mastectomy2 (5/64)
39
Total Mastectomy3 (6/39)

Figure 1

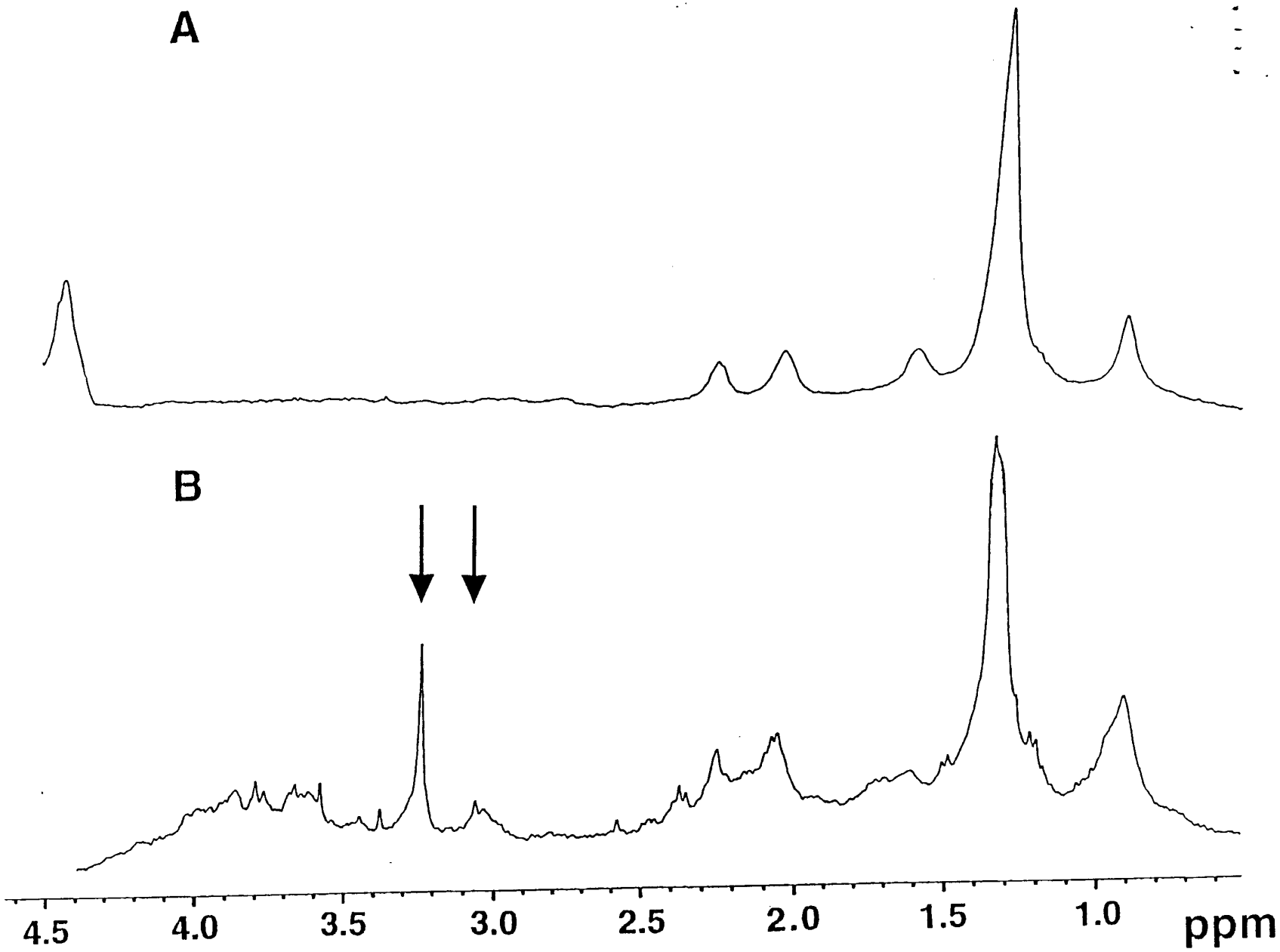

Figure 2

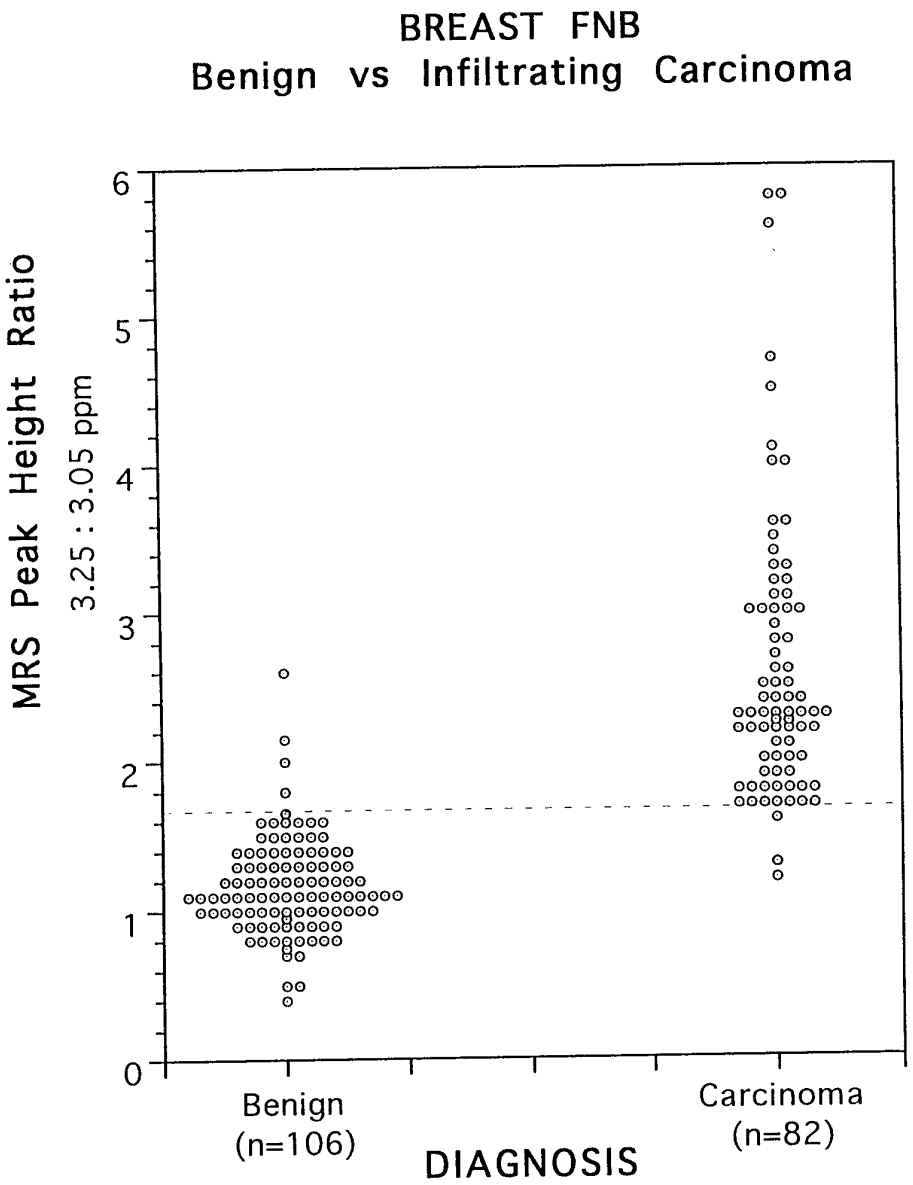
BREAST FNB
Benign vs Infiltrating Carcinoma
MRS Peak Height Ratio
3.25 : 3.05 ppm
6
5
4
3
2
1
0
Benign
(n=106)
Carcinoma
(n=82)
DIAGNOSIS

Figure 3

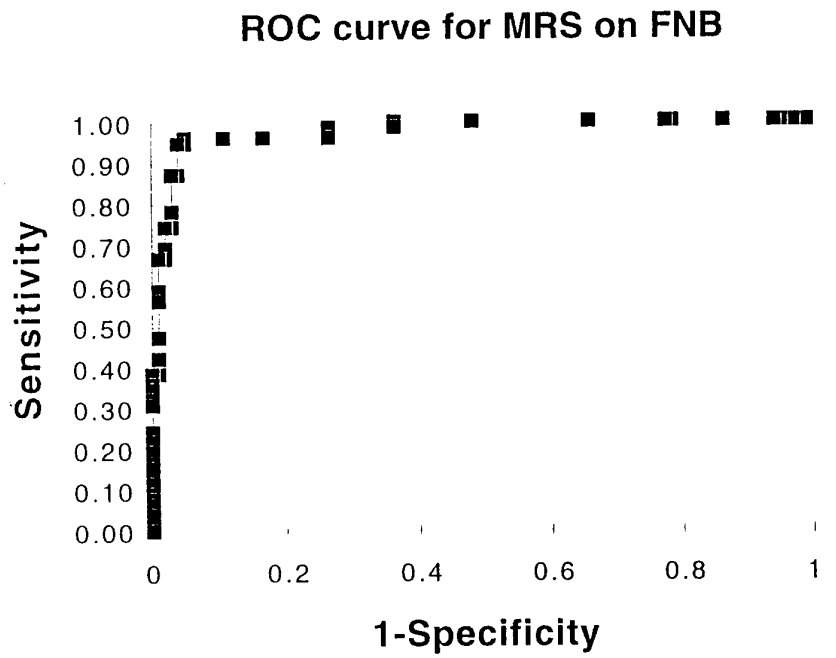
ROC curve for MRS on FNB
Sensitivity
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
0.2
0.4
0.6
0.8
1
1-Specificity

Figure 4

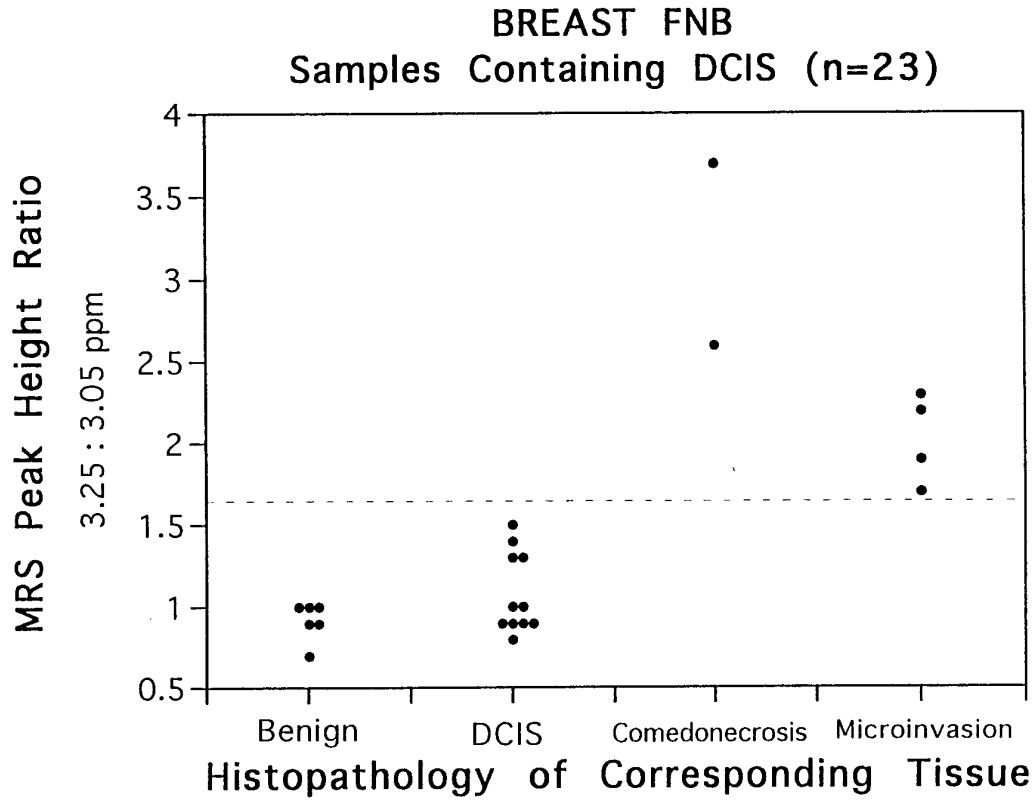
BREAST FNB
Samples Containing DCIS (n=23)
MRS Peak Height Ratio
3.25 : 3.05 ppm
4
3.5
3
2.5
2
1.5
1
0.5
Benign
DCIS
Comedonecrosis
Microinvasion
Histopathology of Corresponding Tissue

Figure 5