Magnetic Resonance to Mange Breast Disease Protocol

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ABBREVIATIONS AND DEFINITIONS OF TERMS

MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
BRCA	Breast Cancer
BRCA 1	Breast Cancer 1 Gene
BRCA2	Breast Cancer 2 Gene
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
miRNA	Micro Ribonucleic acid
CTC	Circulating Tumor Cells
DCE	Dynamic Contrast Enhanced
COSY	Correlated Spectroscopy
DWI	Diffusion Weighted Imaging
PA	Princess Alexandra Hospital
IHBI	Institute of Health and Biomedical Innovation
QUT	Queensland University of Technology
HIRF	Herston Imaging Research Facility
TRI	Translational Research Institute

1. INTRODUCTION

This research project is being conducted in collaboration with The Translational Research Institute, The Centres for MR in health, The Princess Alexandra Hospital, Herston Imaging Research Facility, Hunter Medical Research Institute, Mater Hospital South Brisbane, Mater Hospital Newcastle, Queensland University of Technology and the University of Newcastle.

2. BACKGROUND

2.1 DISEASE

In 2012, there were 15,166 new cases of breast cancer diagnosed in Australia (116 males and 15,050 females)[1] and in 2016, it is estimated that 16,084 new cases of breast cancer will be diagnosed in Australia (150 males and 15,934 females)[1]. In 2016, it is estimated that the age-standardised incidence rate will be 59 cases per 100,000 persons (1.1 for males and 115 for females) [1]. It is estimated that it will become the 3rd most commonly diagnosed cancer in 2016[1] and that the risk of an individual being diagnosed with breast cancer by their 85th birthday will be 1 in 16 (1 in 719 males and 1 in 8 females) [1]. Together, BRCA1 and BRCA2 mutations account for about 20 to 25 percent of hereditary breast cancers and about 5 to 10 percent of all breast cancers[2]. Breast cancers associated with BRCA1 and BRCA2 mutations tend to develop at younger ages than their nonhereditary counterparts[7].

2.2 BRCA1 AND BRCA2 GENE

Inherit BRCA1 and BRCA2 are human genes that produce tumor suppressor proteins[2]. These proteins help repair damaged DNA and, therefore, play a role in ensuring the stability of the cell's genetic material[6]. When either of these genes is mutated, or altered, such that its protein product either is not made or does not function correctly, DNA damage may not be repaired properly[6]. As a result, cells are more likely to develop additional genetic alterations that can lead to cancer[6].

A woman's lifetime risk of developing breast cancer is greatly increased if she inherits a harmful mutation in *BRCA1* or *BRCA2*[2,3]. About 12 percent of Female in the general population will develop breast cancer sometime during their lives[3]. By contrast, according to the most recent estimates, 55 to 65 percent of females who inherit a harmful *BRCA1* mutation and around 45 percent of females who inherit a harmful *BRCA2* mutation will develop breast cancer by age 70 years[4,5].

Specific inherited mutations in BRCA1 and BRCA2 increase the risk of female breast and ovarian cancers, and they have been associated with increased risks of several additional types of cancer. The BRCA1 or BRCA2 mutation can be inherited from a person's mother or father. Each child of a parent who carries a mutation in one of these genes has a 50 percent chance (or 1 chance in 2) of inheriting the mutation. The effects of mutations in BRCA1 and BRCA2 are seen even when a person's second copy of the gene is normal.

3. CLINICAL SIGNIFICANCE

3.1 IMAGING IN BREAST DISEASE

Dynamic contrast enhanced (DCE) magnetic resonance imaging (MRI) is the current method of choice for detecting abnormal breast masses, however its capacity to distinguish benign from malignant lesions remains suboptimal. MR spectroscopy (MRS) has been demonstrated to have the capabilities to improve the diagnostic accuracy by yielding information on the biochemical characteristics of benign versus malignant tumours in vivo[9,10] and to provide information on spread to the nodes from the biopsy of a primary tumour alone[11]. There are two ways of recording this chemical information of breast disease via a fine needle biopsy placed on a pathology slide and then into the magnet, or by placing the patient in a clinical MR scanner.

The chemical information recorded from the biopsy taken from a primary lesion has been shown to identify if the tumor has spread to the nodes without having to biopsy the nodes[11]. MR data collected in vivo identifies the spatial location of the tumor and the pathology[9.] This adds about 15 minutes onto the standard MRI scan time.

We have developed a new MR method two-dimensional (2D) COrrelated SpectroscopY (COSY) method applied in vivo has potential to further improve diagnosis through a detailed inspection of the diagnostic and prognostic chemicals. The COSY method has also been shown to yield information on lipid levels and lipid saturation associated with malignancy[12], and to distinguish lobular from ductal cancers. We have also reported that diffusion weighted imaging (DWI)[13,14] is useful for identification of residual breast carcinoma following neo adjuvant chemotherapy[15] There are now studies reporting the combining of contrast enhanced MRI, and DWI to improve pre-surgical planning and to monitor therapy. The question now arises as to whether combining image guided biopsy to provide MRS information on tumour spread will improve surgical planning. Ductal carcinoma insitu is considered to have four subcategories and the working hypothesis is that these can be identified both on biopsy and in vivo using MRS.

While spectroscopy can be used for diagnostics, it may also provide a better understanding of the risk for cancer development in the high-risk population. We plan to investigate a possible role for breast lipid in tumour development. It is hypothesized that a rogue cancer cell will multiply only in the correct environment. We therefore plan to undertake a pilot study to analyse and compare the breast lipid of females with healthy breasts, BRCA-1 and/or BRCA-2 gene carriers, those with benign, malignant and, DCIS (when available) lesions.

3.2 BIOMARKERS IN SALIVA

In addition, there will be an optional sub-study investigating other biomolecules of clinical relevance that potentially could be found in salvia for BRCA patients (mRNA, miRNA, cells and proteins) [14,15].

DNA methylation in cells (the addition of methyl groups to cytosine residues on the DNA sequence) is an early event that occurs during tumour initiation. In fact, promoter DNA hypermethylation is a more frequent mechanism of gene silencing than genetic mutation[14]. Unlike DNA mutations, DNA methylation abnormalities are reversible by drugs in a laboratory setting and this reversal allows cancer cells to reactivate the silenced genes and produce tumour-suppressor proteins[16,17]. Because DNA methylation normally leads to gene silencing (a negative biological event), a tumour-suppressor protein is not produced and thus, protein detection methods cannot be used. For a diagnostic test to be

implemented clinically, the test will ideally measure a positive event occurring in tumour cells de novo. Therefore, by detecting DNA methylation in cells, one can turn a negative biological event into a positive clinical test. Understanding how abnormal DNA methylation arises in cancer cells, and how this change leads to silencing of genes, is extremely important in the development of treatments that could reverse this process as a strategy to prevent and/or treat cancer.

The preliminary data on DNA methylation in head and neck cancers is very promising as we have identified 3 target genes that are methylated in the saliva collected from head and neck squamous cell carcinoa patients compared to healthy controls. Although, the sensitivity is relatively high, specificity is low. In order to improve the diagnostic power of the salivary DNA methylation marker panel, we will also inlcude other biomolecules of clinical relevance (mRNA, miRNA, cells and proteins) that have been previously documented to be present in saliva of HNSCC patients[17, 19,20] as well as novel biomarkers through a number of highthroughput discovery approaches. The researchers will be collecting blood from study participants to isolate circulating tumour cells to determine whether these can be used as indicators to determine the response to treatment as the literature in this space is inconclusive [19, 20].

Our proposed research programme will firstly focus on whether the same biomarks identified in head and neck cancer patients are present in BRCA patients.

3.3 UNMET CLINICAL NEED

At present there are no early detection / screening tests for breast disease and usually by the time of diagnosis, patients may have developed advanced metastatic cancer. Late diagnosis results in significant mortality, morbidity and health care costs to the Australian government.

4. STUDY AIMS

Aim 1: Examine a biopsy taken from the primary lesion using MRS in the pathology magnet. Ascertain the accuracy of this method to identify if the tumor has spread to the nodes. This will be compared with standard practice sentinel node results. This will require an additional biopsy to be collected by the surgeon.

Aim 2: To undertake bilateral dynamic contrast-enhanced MRI on patients with confirmed clinical indication of breast disease both malignant and benign and those with DCIS, using dynamic/anatomical MRI protocols at 3 Tesla. DCE, MRS data will be compared individually and in combination for sensitivity and specificity.

Aim 3: At the same time as aim 2 undertake in vivo MR spectroscopy to identify the pathology of any lesions identified by MRI.

Aim 4: To ascertain if in vivo MR spectroscopy combined with contrast-enhanced MRI can distinguish DCIS from invasive cancer. This will be achieved by comparison with post-operative pathology.

Aim 5: To develop mathematical classifiers to assess the large volumes of data and produce robust classifiers for objective diagnosis of the different types breast pathology on both biopsy and in vivo data.

Aim 6: Both on biopsy and in vivo determine if MRS can define prognostic factors for the grade 1 cancers which behave aggressively and the with grade 3 cancers that do very well.

Aim 7: Develop systematic approach to improve pre-operative planning by combining MRI, MRS and the current imaging approach.

Aim 8: To examine apparently healthy females 18 to 85 years old who have the BRCA-1 and/or BRCA-2 gene mutations? This is a pilot study to analyze the lipid content and degree of saturation (chemical double bonds in the acyl chains) of breast cancer patients and females with a genetic predisposition to cancer.

Aim 9: Collect saliva samples from participants diagnosed with breast disease to investigate the presence of biomarker panels (DNA, RNA, miRNA, and/or protein and cells) in BRCA patients.

5. RESEARCH METHODS

5.1 TRIAL DESIGN

This is a case controlled study assessing MRS on biopsy or saliva biomarkers can be used to diagnose breast cancer and to determine axillary lymph gland involvement, tumor grade, and hormone receptor status from an analysis of primary tumor alone. If successful this will reduce the need to remove nodes and reduce the incidence of lymphodema. In vivo MRS may improve the diagnostic accuracy of breast MRI, elevating the negative predictive value (identifying benign), positive predictive value (identifying malignant) and confirming nodal involvement by examining the primary tumor alone. The results will demonstrate if MRS can inform physicians of the pathology and spread of tumors preoperatively, and in the future be used to identify tumor margins and assist preoperative planning. If differences are found in the BRCA-1 and/or BRCA-2 populations, this will open a new field of study. No random or stratification is required.

5.2 STUDY PARTICIPANTS

The researchers involved will recruit five participant groups;

(i) Females with confirmed breast lesions (n=100)
(ii) Females with confirmed breast lesions – saliva only (n= approx. 30)
(iii) Females with BRCA1 and/or BRCA2 (n=450)
(iv) Healthy volunteer female group (n= 150)
(v) Healthy volunteer female group – saliva only (n= approx. 30)

5.2.1 VOLUNTEER CONTROL GROUP

Healthy female controls will also be recruited from the general public. The advertisement shall highlight that there shall be no compensation or monetary reimbursement for the volunteers.

Inclusion criteria for healthy controls:

1. Female
2. Age group: 18yrs-85yrs.

3. Be willing to participate for the duration of the trial period.
4. Provide written informed consent.
5. Have no previous medical history of breast disease.
6. Willing to participate in genetic testing.
7. Do not possess BRCA1 or BRCA2 genes.
8. Be available for follow-up if and when needed.
9. Be willing to provide contact information for their general practitioner.

Exclusion Criteria for healthy controls:

1. Previous history of breast disease.
2. Presence of BRCA1 or BRCA2 gene.
3. Current or suspected pregancy.
4. Males.
5. Females under the age of 18 years.
6. Those who are unable to provide informed consent.

5.2.2 VOLUNTEERS WITH CONFIRMED BREAST LESION(S)

A group of 100 participants will be recruited through clinics at the Princess Alexandra Hospital, Mater Hospital South Brisbane, Mater Hospital Newcastle or through the general population.

Inclusion criteria for breast lesion participant group:

1. Female
2. Age group: 18yrs-85yrs.
3. Be willing to participate for the duration of the trial period.
4. Provide written informed consent.
5. Be scheduled for surgery.
6. Willing to donate breast tissue/lesion sample.
7. Willing to participate for the duration of the trial period.
8. Available for follow-up appointments if required.

Exclusion Criteria for breast lesion participant group:

1. Current or suspected pregancy.
2. Females under the age of 18 years.
3. Those who are unable to provide informed consent.

4. High risk of poor compliance to participate with study requirements or follow-up as assessed by the investigator

5.2.3 BRCA1 AND/OR BRCA2 PARTICIPANT GROUP:

A group of 450 participants will be recruited through clinics at the Princess Alexandra Hospital, Mater Hospital South Brisbane, Mater Hospital Newcastle or through the general population.

Inclusion criteria for breast lesion participant group:

1. Female
2. Age group: 18yrs-85yrs.
3. Willing to participate in genetic testing.
4. Presence of BRCA1 or BRCA2 gene.
5. Be willing to participate for the duration of the trial period.
6. Provide written informed consent.
7. Willing to participate for the duration of the trial period.
8. Available for follow-up appointments if required.
9. Willing to provide contact details for general practitioner and specialists involved in participants care.

Exclusion Criteria for breast lesion participant group:

1. Current or suspected pregancy.
2. Females under the age of 18 years.
3. Those who are unable to provide informed consent.
4. High risk of poor compliance to participate with study requirements or follow-up as assessed by the investigator.

5.2.4 SALIVA BRCA GROUP

Approximately 30 participants will be recruited through the Breast Clinic at the Princess Alexandra Hospital.

Inclusion criteria for saliva control group:

1. Age group: 18yrs-85yrs.
2. Willing to provide saliva and/or blood samples for the purpose of the sub-study.
3. Provide written informed consent.
4. Confirmed history of breast cancer.

Exclusion Criteria for Patient Group:

1. Current illness that will interfere with the collection of saliva and/or blood samples

2. High risk of poor compliance to participate with study requirements or follow-up as assessed by the investigator

5.2.5 SALIVA HEALTH CONTROL GROUP

Inclusion criteria for saliva control group:

1. Age group: 18yrs-85yrs.
2. Be willing to provide saliva and/or blood samples for the purpose of the sub-study.
3. Provide written informed consent.
4. Medical History: No prior history of any cancer. Patients with family history of cancers however can be included.
5. No previous irradiation.
6. No history of Sjogren syndrome or other pathology limiting saliva production.
7. No fever or signs and symptoms suggesting active infection/illness on the day of saliva donation.
8. Substance use: Volunteers with history of smoking, active smokers and non-smokers as well as drinkers and non-drinkers will be recruited as long as they have no current dysplastic lesion or cancer.
9. Available for follow-ups if and when needed.

Exclusion criteria for healthy controls:

1. Previous history of any type of cancer.
2. Presence of leukoplakia and/or erythroplakia.
3. Previous irradiation.
4. Sjogren syndrome or other pathology limiting saliva production.
5. Signs and symptoms of fever which may suggest active infection/illness on the day of saliva donation.
6. High risk of poor compliance to participate with study requirements or follow-up as assessed by investigator.

5.3 CONSENT

Those participants who wish to participate or are recruited will be provided the patient information and consent form prior to participation. The participant will be given time to read through the consent and ask their referring specialists any questions. The participant can also ring the Clinical Principal Investigator or the nurse project manager listed on the patient information and consent form for further information.

5.4 WITHDRAWING FROM STUDY

Participants have a right to withdraw from the study at any point. The patient information and consent form states that “If you decide to withdraw, please notify a member of the research team about your

decision. Your decision will not affect your routine treatment, your relationship with those treating you or your relationship with the site. Any identifiable personal information will be destroyed while de identifiable data will be used for the study purpose only".

6. DATA COLLECTION

6.1 IMAGING DATA

Scanner:
Data will be obtained using the Siemens 3T Prisma (Siemens AG, Erlangen, Germany).

Imaging:
For anatomical localisation and voxel placement a three dimensional high resolution MPRAGE (Magnetisation-Prepared Rapid Acquisition with Echo Gradient) scan will be obtained (TR/TE=2530/1.7 ms, 12 degree flip angle, FOV= 256x256mm, voxel size 1x1x1mm, NEX 4, acquisition time 6 minutes).

1D Spectroscopy:
Single voxel PRESS sequence will be obtained using the following parameters: TR 1.5s; TE 30ms; voxel size 2×2×2cm=8cm^3; 96 averages. One-dimensional spectra will be obtained.

2D Spectroscopy:
Will be obtained in the ACC using the following acquisition parameters: RF carrier frequency at 2.0 ppm; TR 1.5s; water suppression; spectral width of 2000Hz; increment size of 0.8 ms in 96 t1 increments giving an indirect spectral width of 1250Hz; 12 averages per increment; and 1024 data points. Scan time will be approximately 30 minutes.

Diffusion Tensor Imaging (DTI):
35-directions scan; TR: 5520ms; TE: 89.5ms; FOV: 210×180; slice thickness 1.25mm; Multiband = 2; IPAT = 2; b-value = 0, 1000 and 3000 s/mm^2.

Resting state fMRI:
TR/TE = 2390/24ms, FOV = 260mm×260mm, flip angle = 90°, slice thickness = 3.0mm without gap, interleaved scanning, 47 slices covering the whole breast, 250 volumes acquired in 10 minutes.

Post-Acquisition Analysis:
One-dimensional spectroscopy will be processed using LC-Model. LC-Model is an automated package that uses a time domain-fitting loop. The best fit is found by varying a basis set if concentration-calibrated model spectra of individual metabolites. LC – Model provides identification and estimation of the absolute concentrations of metabolites. The average metabolite ratios will be compared between each group and tested using a t-test or Mann-Whitney if the data are not normally distributed.

Statistical classification algorithms:
Raw one dimensional spectroscopy data will be input into a comprehensive set of statistical classification algorithms. Before data is analysed it is post-processed using the following steps: 1st spectral alignment; water removal; apodization; phase correction; baseline removal; and 2nd spectral alignment. Feature extraction is performed using a wavelet-based transform, undertaken on

the entire spectrum. Candidate biomarkers are compared using a two-sided, equal variance, student t-test and are considered statistically if $p < 0.01$. Extracted features can then be correlated to clinical measures that have been collected throughout the project such as the McGill pain questionnaire and the State-Trait Anxiety Inventory. The clinical measures will be compared to features using a spearman correlation co-efficient. Biomarkers that have been identified can be tested to determine their sensitivity and specificity.

Two-Dimensional COSY:
Raw 2D spectroscopy is transferred from the scanner to Matlab. Within Matlab the signal is combined from multiple elements, rows concatenated into a 2D matrix and reformatted. The resulting 2D file is now processed and analysed using Felix, specialised 2D nuclear MR processing software. In Felix each prominent diagonal and cross peak is selected and integrated to determine the peak chemical shift; intensity and volume. These values are standardised (using creatine diagonal cross peak at 3.02 ppm) and can then be compared statistically for each group.

6.2 SALIVA DATA COLLECTION

The following data items will be collected for each of the participants recruited from the Breast Clinic at the Princess Alexandra Hospital. This information will be obtained from the refering specialist and the participant.
Demographic Information:

- Age
- Gender
- Family history of cancer

Clinical Information:

- Confirmation of breast cancer diagnosis

7. SAMPLE COLLECTION AND ANALYSIS

7.1 BREAST LESION

A sample of the breast lesion is taken using fine needle aspiration (FNA). It is the least invasive method of biopsy and it usually leaves no scar. The participant will be lying down for this procedure. First, an injection of local anaesthesia (lignocaine) is given to numb the breast. The surgeon uses a thin needle with a hollow center to remove a sample of cells from the suspicious area. In cases where the lump cannot be felt, the surgeon or radiologist may need to use ultrasound equipment to guide the needle to the right location. The doctor then inserts the hollow needle to remove the cell sample.

The sample will then undergo analysis using Spectroscopy as it may provide additional information on the characteristics of the lesion. This will be completed prior to the participant's MRI and MRS scans.

7.2 BLOOD COLLECTION

Those who have an unconfirmed BRCA genetic status will be asked to provide blood for analysis. Blood collection will be completed with either the relevant hospital's blood collection services or at the

Clinical Research Facility. The amount of blood collected for BRCA genetic testing is 16ml. Genetic testing will be completed through Pathology Queensland.

For those participants who are willing to also donate blood to the saliva sub-study is 30ml. As a proof of concept in a preliminary study, the study will require collection of blood samples from BRCA patients with advanced stage breast cancer. The pathology will be analysed at QUT IHBI. Cerculating tumor cells (CTC) will be isolated[21] using the CellSearch (Veridex, USA), Circulating rare cells (CRC) Technology (ScreenCell, France) or the Carcinoma Cell Enrichment Kit (Miltynyl Biosystems, Germany). Enrichment of disseminated carcinoma cells from peripheral blood will be performed by positive selection of cytokeratin 7/8 expressing cells. As cytokeratins are expressed in a tissue-specific manner by epithelial cells, the research will examine the possible use of cytokeratin 8 and 19 as these are widely expressed by mucosal epithelial tumours[21]. Total RNA,miRNA will be extracted from the isolated CTCs followed by CDNA synthesis and PCR amplification to determine the presence of CTCs in peripheral bloods.

7.3 SALIVA SAMPLES

Saliva samples will be collected from study participants and the salivary flow rates will be determined (this information is important to determine the volunteers who are suffering from dry-mouth syndrome). The salivary flow rate determination is a specific procedure where salivation is induced and the amount of saliva expressed is compared to a standard. Subjects will be requested to refrain from consuming food or drink (except water) at least 1 hour prior to donating saliva if feasible. This includes also refraining from chewing gum. Saliva samples shall be collected using the 'drool' method. Participants will be asked to provide approximately 1-5mls of saliva into a specimen cup.

8. SPECIMEN STORAGE

Saliva and blood samples used in the saliva sub-study will be transported from the point of collection to the Institute of Health and Biomedical Innovation, Queensland University of Technology or collaborators according to Australian Government Department of Health and National Pathology Advisory Council reqirements. All other specimens will be discarded upon analysis.

9. ETHICS APPROVAL

This study will be performed under the regulatory approval of the Metro South Human Research Ethics Committee.

10. MONITORING AND AUDITS

The Principal Investigators alongside the Translational Research Institute will permit project-related monitoring, audits and regulatory inspections, providing direct access to source data and documents. This may include, but not limited to, review by the HREC committees involved and institutional governance review bodies.

11. QUALITY CONTROL

This study will be conducted in accordance with the National Statement of Ethical Conduct in Human Research, The Australian Code for The Responsible Conduct of Research, relevant policies and procedures and under the guidelines of ICH GCP as annotated by the Australian Therapeutic Goods Administration.

12. DISCLOSURE AND PUBLISHING

The results of the study will be presented at medical and scientific meetings and will be published in peer-reviewed medical or scientific journals

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