Improving Breast Health: The Use of Hormonal Combinations to Reduce Mammographic Breast Density and the Utility of Shear Wave Elastography as a Biomarker of the Effects of these Interventions

by

Daniella Dougherty

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This thesis is dedicated to my late father

Gavin James Dougherty

You would have been the only Dougherty excited to read this thesis

And I really wish you could have

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Summary

Breast cancer is a major health concern in both developed and developing countries. Although there are many factors that can increase one's risk of breast cancer; mammographic breast density is labelled as the greatest, modifiable risk factor of breast cancer. So much so, it has been used as a surrogate endpoint in breast cancer intervention trials.

Mammographic breast density does have its flaws, especially when being used to monitor therapeutic interventions used to reduce breast cancer risk in clinical practice or clinical trials. To name a few, mammographic breast density responds to interventions slowly, taking up to one and a half years to show significant changes within the breast tissue. Mammographic breast density also requires women to have mammograms, which are not the most pleasant experience, and can lead to women not attending breast cancer screenings or high attrition rates in breast cancer trials. Also, the quantification of mammographic breast density itself can occur in a subjective manner, which can lead to substantial assessor influence on the results.

There appears to be a gap in the literature for a biomarker for mammographic breast density, which responds (in a timelier manner) to treatments aimed at reducing mammographic breast density and subsequently breast cancer risk. This thesis aimed to investigate if breast elasticity, as measured by shear wave elastography, is a viable biomarker for mammographic breast density, which can be used in clinical practice or research.

This thesis consisted of two main focuses. Firstly, determining if elasticity responds to treatment that also alter mammographic breast density. Secondly, determining a standardised protocol to objectively measure whole breast elasticity, using the SuperSonic™ Imagine Aixplorer® ShearWave™ elastography machine. The first focus consisted of three clinical trials. The first, an analysis of a patient database of women who used HAVAH*T+Ai*™ to reduce mammographic breast density. The second, a three-month, open-labelled, pharmacokinetic sub-study, determining if breast elasticity changes occurred with HAVAH*T+Ai*™, as it was shown to reduce mammographic breast density in the initial study.

Lastly, a 12-month, open labelled, clinical trial of a combination of a selective androgen receptor modulator and an aromatase inhibitor. Analysing the effect of this combination on mammographic breast density and breast elasticity, enabling the determination of consistency with the elasticity response with two interventions that were shown to reduce mammographic breast density. Correlations between breast elasticity and mammographic breast density variables are also discussed with the potential favourable uses of these in clinical and research settings.

The second focus included two studies. The first, an analysis of what shear wave elastography protocols created the most precise data. Secondly, an eight-week study, determining the effects of the hormonal changes of the menstrual cycle, patient position, and repeat measurements of breast elasticity, using shear wave elastography. Using this data to create a description of a standardised protocol to use when using shear wave elastography to measure whole breast elasticity, which will aid and standardised future use and research of breast elasticity as a biomarker for mammographic breast density and breast cancer risk.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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Conference Abstracts and Awards During Candidature

Conference Abstracts

The potential utility of Shear Wave Elastography as a biomarker of the effects of interventions targeted at reducing Mammographic Breast Density | *Accepted for the 9th International Breast Density & Cancer Risk Assessment Workshop, June 2019*

Anastrozole and enobosarm (GTx-024): the effect of an aromatase inhibitor and selective androgen receptor modulator on Mammographic Breast Density and breast pain in premenopausal women | *Accepted for the 9th International Breast Density & Cancer Risk Assessment Workshop, June 2019*

Improving breast health: The potential utility of Shear Wave Elastography as a biomarker of the effects of interventions targeted at reducing breast density and inflammation | *Presented at the University of Adelaide 12th Annual Florey International Postgraduate Research Conference, 2018*

Awards

Adelaide Medical School Prize at the 12th Annual Florey Postgraduate Conference
The MERCK Prize at the 12th Annual Florey Postgraduate Conference

Shear Wave Elastography Preface

Shear wave elastography is a new form of medical imaging which uses shear wave acoustic waves, induced by the radiation force of a focussed ultrasonic beam, to image and characterise tissue structures (Sarvazyan, Rudenko et al. 1998). Further details pertaining to the science behind shear wave elastography are provided in Section 1.5.2 within this thesis.

This preface will provide a description of some of the key features of the equipment and a glossary of terms specific to this piece of equipment. The purpose of this preface and glossary is to be used as an introduction to the machine and be used as a reference as required by the reader.

The machine used within this thesis was the SuperSonic™ Imagine Aixplorer®, which can provide real-time ShearWave™ Elastography (SWE), pictures in Figure 1.

Figure 1: SuperSonic™ Imagine Aixplorer® ShearWave™ Elastography Machine

As this is an ultrasound machine, a transducer head is required to be used; for this research the SuperLinear™ SL15-4 linear transducer was used, pictured in Figure 2. This transducer head had a bandwidth of 2-10 megahertz (MHz) and can be used for breast ultrasounds, as well as for abdominal, musculoskeletal, paediatric, thyroid, vascular and general applications.

Figure 2: SuperLinear™ SL15-4 Linear Transducer Head

A generic, water-based ultrasound gel was also used on both the skin and on the transducer head. The ultrasound gel and the ultrasound gel on the transducer head are pictures in Figure 3 and 4, respectively.

Figure 3: Generic ultrasound gel

Figure 4: Ultrasound gel on the Linear transducer head

Shear Wave Elastography Glossary

B-Mode Ultrasound: B-mode ultrasound is a widely accepted method of ultrasound, where a linear array of transducer simultaneously scans a place through the body. This scan can be viewed as a two-dimensional image on the screen (Carovac, Smajlovic et al. 2011). On the Supersonic™ Aixplorer® SWE machine the B-mode ultrasound is displayed simultaneously with the SWE on the Aixplorer® screen, as pictured in Figure 5.

ShearWave™ Elastography (SWE mode): The SWE mode uses shear waves and measures the speed at which they travel through the tissue and displays information about tissue elasticity. It is displayed as an easy to interpret colour-coded image on the Supersonic™ Aixplorer® SWE machine, as pictured in Figure 5. This elasticity can also be viewed as a quantitative value, with the local estimation of tissue elasticity, as either kilopascal or meters per second.

Region of Interest (ROI): The region of interest is the area of the ultrasound output that is the focus of the image (the focal zone), as pictured in Figure 5. For the Supersonic™ Aixplorer® SWE system, the ROI is within a box on the image, that can be moved or resized on the image.

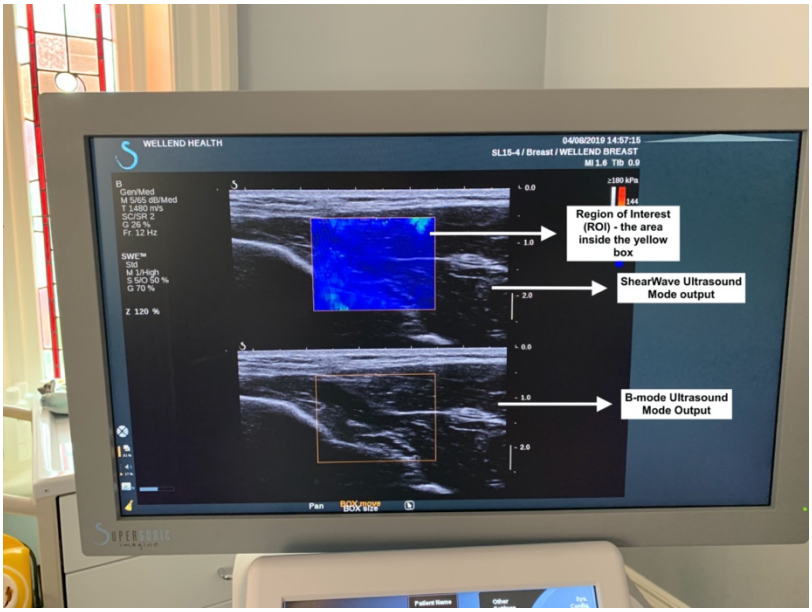

Figure 5: SuperSonic™ Imagine Aixplorer® SWE machine output with the ShearWave Elastography mode and B-mode ultrasound simultaneously visible on the machines output screen. The Region of Interest is the focal point of the ShearWave Ultrasound

The Quantification Box (Q-Box™): The commonly used term for the quantification box is the Q-Box™. This is a customisable circle that you place on the image to accurately quantify the elasticity (also called the stiffness) of an area. The Q-Box™ can be resized and/or moved across the image and anchored to display to elasticity within that selected area. A singular 10-millimetre Q-Box™ is pictured in Figure 6.

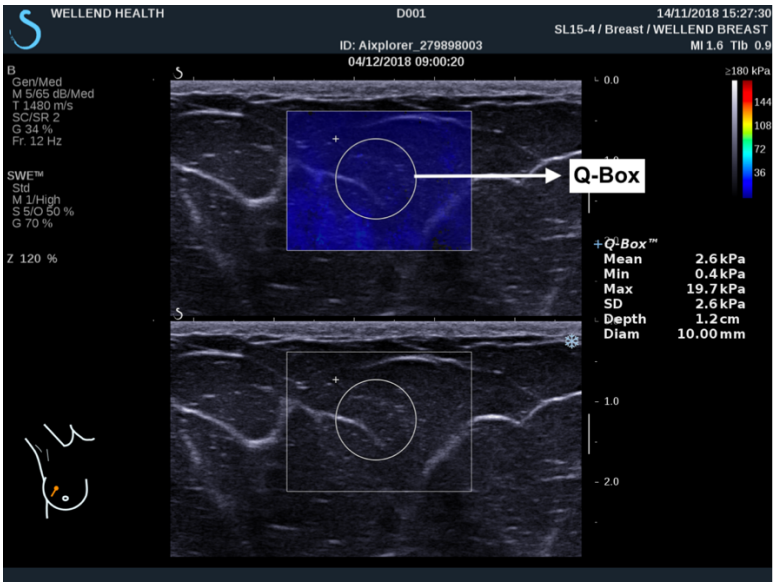

Figure 6: The Q-Box™ displayed on both the SWE mode and the B-mode ultrasound output

Q-Box™ Ratio: The Q-Box™ ratio is similar to the Q-Box™ but it allows you to compare the elasticity of two areas of the same image. Within this thesis, one circle, that again could be resized or moved, was placed on an area of the image that was fibroglandular tissue and one was placed on an area of fatty tissue. The elasticity for each subtype of tissue are presented on the screen.

Q-Box™ Trace: This is when you manually trace a Q-Box™ on the SWE image, it provides the elasticity values within the traced area. The area can be traced using the trackball on the Aixplorer® SWE machine or with the stylus attached. The Q-Box™ trace is pictured in Figure 7.

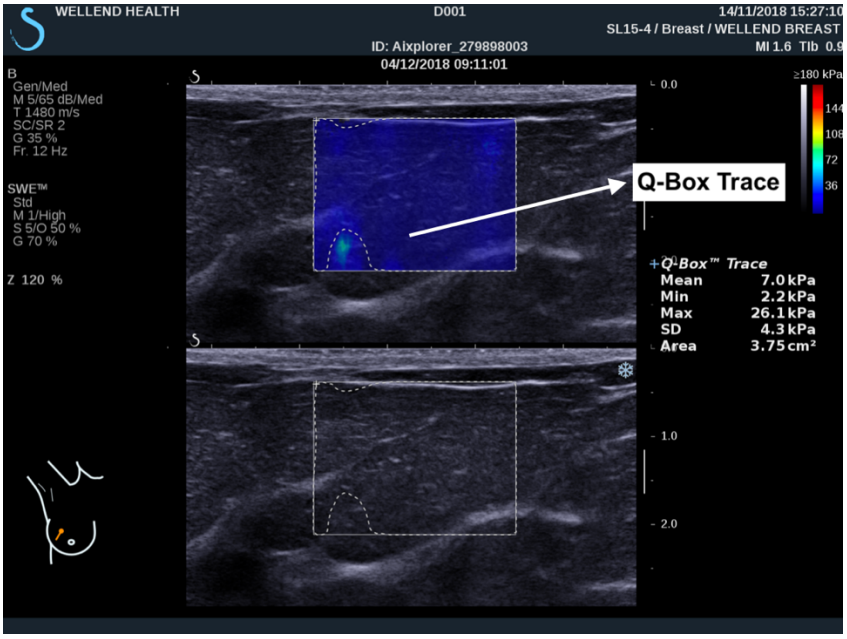

Figure 7: The SWE mode image with the Q-Box™ trace function used to trace and create a custom shaped Q-Box™ to include the area within the ROI for which will be included in the elasticity

Export: The data that was generated from the shear wave elastography image can be exported from the machine as either a comma separated value (CSV) excel files, DICOM files or as generated reports with the relevant images selected, pictured in both Figure 8 and 9.

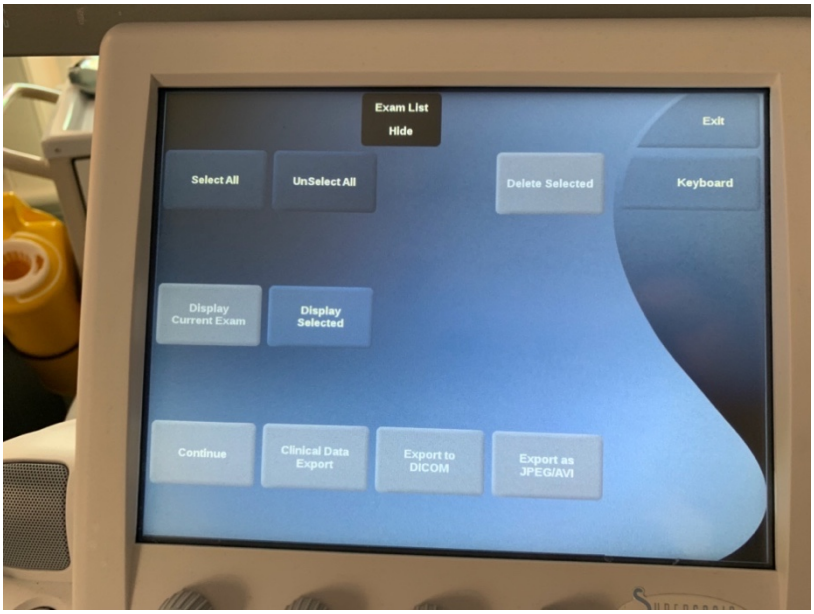

Figure 8: The screen on the SuperSonic™ Imagine Aixplorer® SWE machine that provided the options for exporting the data

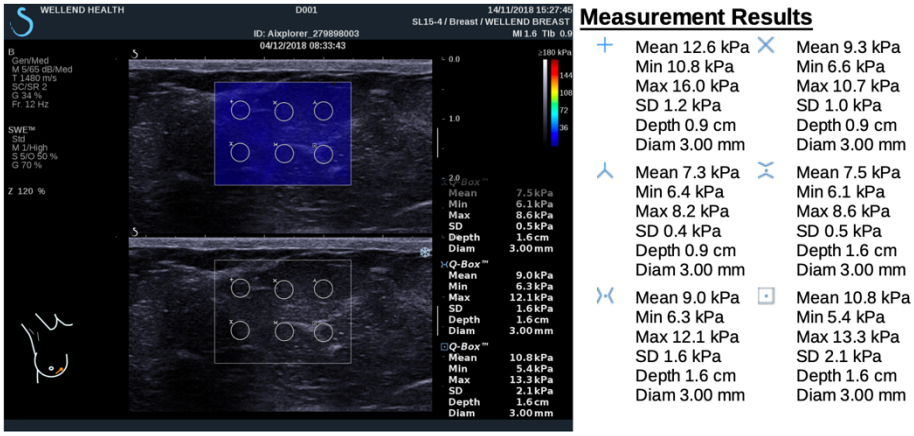

Figure 9: A sample of the reports generated by the machine that can be exported with both the image and the quantitative data

Black Hole: Areas of the Shear Wave Image where the shear waves have not propagated through the tissue and as a consequence have no elasticity data.

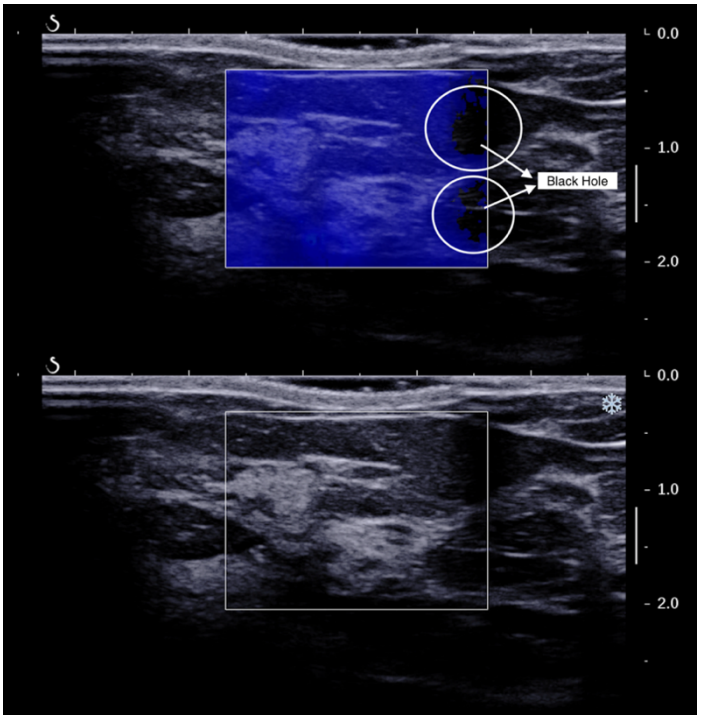

Figure 10: A Shear Wave ultrasound image with a black hole present

Artefacts: Areas on the colour elasticity image that might not be a representation of the mechanical properties of the breast tissue but rather an issue with the SWE. The areas in the image that have artefacts have extremely high elasticity values, which do not correspond to any structure on the B-Mode image.

Abbreviations

%BD	Per cent breast density
%FV	Per cent fibroglandular Volume
%VBD	Percentage volumetric breast density
5α-DHT	5α-dihydrotestosterone
6 x 3mm Q-Box™	Six, three-millimetre Q-Box™
6 x 6mm Q-Box™	Six, six-millimetre Q-Box™
ADH	Atypical ductal hyperplasia
AE	Adverse event
Ai	Aromatase Inhibitors
ALH	Atypical lobular hyperplasia
ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate-activated protein kinase
ARFI	Acoustic radiation force impulses
BBD	Benign breast disease
BC	Breast cancer
BD	Breast density
Bi-RADs	Breast imaging reporting and data systems
BMI	Body mass index
Boyd's SCC	Boyd's six class categories
CCHT	Continuous combined hormone therapy
CCS	Case control study
CI	Confidence Interval
Cm	Centimetre
Cm^3	Cubic centimetre
CS	Cohort study
CV	Coefficient of variation
DHEAS	Dehydroepiondrosterone sulphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
E/A Ratio	Oestrogen/androgen Ratio
E_1	Oestrone
E_2	Oestradiol
ECM	Extracellular matrix
EOS	End of study
FAI	Free androgen index
FGV	Absolute volume of fibroglandular tissue
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
Gtx-024	Enobosarm
HAVAH*T+Ai*™	HAVAH proprietary limited testosterone and anastrozole
HER2	Human epidermal growth factor receptor 2
HREC	Human research ethics committee
HRT	Hormonal replacement therapy

IBIS-I	International breast cancer intervention study I
ICMD	International consortium on mammographic density
IMI	Image mean index
Kg	Kilogram
kPa	Kilopascal
LCIS	Lobular carcinoma in situ
LH	Lutenizing hormone
LVI	Lymphovascular Invasion
m/s^{-1}	Meters per second
MBD	Mammographic breast density
MDA	Malondialdehyde
MDEST	Mammographic density estimation
mg	Milligram
mGY	Milligray
ml	Millilitres
MMP-3	Metalloproteinase-3
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance Imaging
MRIV	Magnetic resonance Imaging Volume
MRS	Menopause rating scale
N/m^2	Newton per square meter
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
Pa	Pascal
PMD	Percentage mammographic density
PT	Preferred term
RAS	Restricted analysis set
RCT	Randomised controlled Trial
ROI	Region of interest
SARM	Selective androgen receptor modulator
SCC	Six class categories
SEER	National Cancer Institute age-specific surveillance, epidemiology and end results program
SERM	Selective oestrogen receptor modulators
SHBG	Sex hormone binding globulin
SOC	System organ class
SWE	Shear wave elastography
T	Testosterone
TBV	Total breast volume
TDLUs	Terminal duct lobular units
TFV	Total fibroglandular volume
TIMP	Tissue inhibitor of metalloproteinases
TIMP-3	Tissue inhibitor of metalloproteinases-3
TM	Trademark
u/L	Unites per litre
VAS	Visual analogue scale
VPD	Volumetric per cent density

X^2	Chi-squared statistic
α	Alpha
β	Beta

Chapter 1 Introduction and Background

1.1 Introduction

Breast cancer is one of the most prevalent types of cancer among women and affects women of both developed and developing countries. While there has been great progress into diagnostic and treatment techniques, there remains a substantial morbidity and mortality burden of this disease with more than two million cases diagnosed and more than 600,000 patients dying from this condition worldwide annually (Bray, Ferlay et al. 2018). There has been a great deal of research undertaken to establish the many risk factors that have been linked to an increased risk of developing breast cancer; and that there is heterogeneity in regard to the risk factors associated with breast cancers of different subtypes (oestrogen or progesterone receptor positive or Human Epidermal Growth Factor Receptor 2 (HER2) tumours) (Althuis, Fergenbaum et al. 2004, Ma, Bernstein et al. 2006, Yang, Chang-Claude et al. 2011). Broadly, McPherson, Steel et al. (2000) summarised these risk factors which include;

- Increasing age (up to the onset of menopause)
- Geographical location
- Early menarche
- Late menopause
- Family history
- Age at first birth (with nulliparity and late age of first birth increases the risk)
- Previous benign breast disease (BBD)
- Exposure to radiation

Furthermore, lifestyle factors may also contribute to an increased risk of breast cancer, these factors include;

- Diet (dietary fat intake is correlated with an increased incidence of breast cancer)
- Obesity in postmenopausal women (among premenopausal women obesity is associated with a reduced incidence of breast cancer)
- Alcohol consumption
- The use of oral contraceptives

- Hormonal replacement therapy (HRT), with the risk of breast cancer appearing higher in combined oestrogen and progesterone combinations (McPherson, Steel et al. 2000).

In addition to the risk factors listed above, in recent years, it has been discovered that mammographic breast density (MBD) is a major independent and modifiable risk factor for breast cancer. With previous research predicting that a third of breast cancers could be linked to a woman having highly dense breasts (Boyd, Martin et al. 2010).

1.2 Mammographic Breast Density

Mammographic breast density refers to the percentage of dense tissue compared to fatty tissue within a woman's breast. The fatty tissue is radiologically lucent and appears dark on a mammogram, while in contrast, epithelium and stroma are radiographically dense and appear light on the mammographic breast image. Mammographic breast density is highly variable and differs significantly amongst individuals of the same age and race (Ingleby and Gershon-Cohen 1960). An association between the mammographic parenchymal pattern of the breast and breast cancer risk was initially proposed in 1976 by Dr John Wolfe (Wolfe 1976), and this has led to advancing research within this field.

Boyd, Lockwood et al. (1998) measured the proportions of mammographic images occupied by radiographically dense tissue. They found, without exception, a compelling association between increasing densities and increasing risk of breast cancer. Furthermore, McCormack and dos Santos Silva (2006) conducted a meta-analysis of 42 studies which included 14,000 cases and 226,000 non-cases; finding that there was a consistent relationship with higher percentage mammographic density (PMD), which is another method of quantifying breast area, and an association with breast cancer risk. In addition, there are further studies revealing that breast cancers arising from areas of high MBD are more frequently related to factors suggestive of a poorer prognosis, including large tumour size, higher histologic grade, lymphovascular invasion (LVI) and the cancer being discovered at a more advanced stage, compared to those arising within an area of low MBD tissue (Aiello, Buist et al. 2005). Although some of the previously mentioned risk factors for breast cancer are associated with or can influence MBD; after adjustment for the effects of these factors, MBD remains an

independent risk factor for the development of breast cancer (McCormack and dos Santos Silva 2006, Boyd, Martin et al. 2010, Kerlikowske, Shepherd et al. 2005).

1.2.1 Histology of the Tissue Associated with Mammographic Breast Density

Mammographic breast density is a trait that is linked to the variation in the fibro-epithelial architecture of the breast (Boyd, Lockwood et al. 1998). The histology of the tissue of differing MBD's has been studied, utilising both surgical biopsies and mastectomy specimens. These studies demonstrated that epithelial and stromal proliferation was associated with increased MBD (Wellings and Wolfe 1978, Bland, Kuhns et al. 1982, Bright, Morrison et al. 1988, Urbanski, Jensen et al. 1988, Bartow, Pathak et al. 1990, Boyd, Jensen et al. 1992). However, the samples examined within these studies were taken from women with known or suspected breast disease. It needs to be noted that this may not be an appropriate sample to represent the general population of women with high MBD. Furthermore, Li, Sun et al. (2005) examined breast tissue samples obtained from forensic autopsy and found that tissue with high PMD was associated with a greater proportion of collagen, a greater area of glandular structures and a greater nuclear area of both epithelial and non-epithelial cells when compared to tissue of a lesser PMD. This larger proportion of collagen accounted for 29% of the variance in the PMD with other tissue measurements (area of glandular structures and area of epithelial and non-epithelial cells) accounting for between 4% and 7% of the variance in PMD (Li, Sun et al. 2005).

Additionally, Turashvili, McKinney et al. (2009), using the same tissue samples as the Li, Sun et al. (2005) case-series, found that premenopausal women who had high MBD at a younger age demonstrated pre-neoplastic cellular changes, with the presence of columnar cell lesions being associated with high MBD. Columnar Cell Lesions having been proposed to represent the earliest identifiable histological, but non-obligatory precursor, of low-grade breast carcinomas (Sewell 2004, Simpson, Gale et al. 2005). Additionally, tissues with high MBD share key histological features with stromal components of malignant breast lesions, specifically low adipocytes and high extracellular matrix (ECM) content (DeFilippis, Chang et al. 2012).

Furthermore, Lisanti, Reeves et al. (2014) reported that there are similarities in the gene expression in high MBD fibroblasts and those seen in cancer associated fibroblasts. In particular, high density up-genes showing associations with gene sets related to cancer, the stress response, inflammation, stemness, and signal transduction. In their study, Lisanti, Reeves et al. (2014) demonstrated that there was a strong resemblance between high MBD fibroblasts and human tumours, including breast cancers with these results potentially reflecting functional similarities between cancer associated fibroblasts and high MBD fibroblasts; which are a major component of the tumour stroma in most solid tumours.

1.2.2 Measuring Mammographic Breast Density

Since MBD has been identified as an important breast characteristic linked to breast cancer, more advanced methods and technologies have been developed to quantify this characteristic. To date, three different broad methodological categories exist to quantify MBD, these being manual, semi-automated and fully automated methods.

1.2.2.1 Manual Methods

Dr John Wolfe, as previously mentioned in Section 1.2, was the first individual credited as linking the breast parenchymal pattern to breast a woman's breast cancer risk. To do this, originally Wolfe applied a method, using both qualitative as well as quantitative criteria, to classify and categorise the parenchymal patterns of the breast. Wolfe (1976) created the following descriptions of these categories;

- N1 category – this refers to breast parenchyma, which is composed primarily of fat, with at most, a small amount of dysplasia; no ducts are visible for this category
- P1 category – this refers to parenchyma composed chiefly of fat, with prominent ducts in the anterior portion to one fourth the volume of the breast; also, maybe a thin band of ducts extending into a quadrant
- P2 category – this refers to severe involvement with dysplasia, with prominent ductal patterns occupying more than one-fourth the volume of the breast
- DY category – this refers to severe involvement with dysplasia, often obscuring an underlying prominent ductal pattern

As this method of classification is subjective, it has resulted in inconsistencies in the application of these categories when analysing mammographic images. Oza and Boyd (1993) conducted a review to determine the reliability of the Wolfe classification categories; they found that the inter-observer agreement was 52% to 97% and that there was an intra-observer agreement of 69% to 97%. In terms of monitoring changes in MBD, the variability that is associated with this method leads to unreliable results and for this reason, in the early 1980s attempts began to develop a reproducible, quantitative method of assessing and classifying MBD (Boyd, O'sullivan et al. 1982).

A further method of manually classifying MBD is using a visual estimation of the percentage of the breast occupied by dense tissue, with several different models being utilised each with differing variables and categories (Boyd, O'sullivan et al. 1982, Boyd, Byng et al. 1995, Vachon, King et al. 1999). In the United States of America, the Breast Imaging Reporting and Data Systems (BI-RADs) was developed to standardise the reporting terminology of the mammography assessment categories (American College of Radiology 1998). The BI-RADs method is based on qualitative visual assessments and can be highly influenced by the reviewer as the density classification is assigned by the radiologist based on the visual inspection of the image (Jeffers, Sieh et al. 2016). This method distinguishes four categories;

A. Almost entirely fatty
B. Scattered areas of fibroglandular densities
C. Heterogeneously dense
D. Extremely dense

The BI-RADs method has a moderate inter-observer agreement, with a kappa coefficient value of 0.43 to 0.59 (Kerlikowske, Grady et al. 1998, Berg, Campassi et al. 2000). The BI-RAD system, however, was not intended to provide a quantification of MBD, it was included in the mammography report to inform the referring physician of the impact of the sensitivity and the interpretation of the image based on the level of breast density (Harvey and Bovbjerg 2004). This impact on the mammogram interpretation is due to a decreased level of sensitivity to detect lesions on mammograms with high BI-RAD categorisation (Harvey and Bovbjerg 2004), which is a phenomenon called masking; which occurs when the breast lesions are hidden by the dense tissue on the mammogram as they both appear white on the

imaging. The BI-RAD scale was also not intended to quantitate changes in the breast tissue density over time.

1.2.2.2 Semi-automated Methods

To reduce some of the limitations faced by the previously discussed manual visual estimation methods, more consistent semi-automated, computer-assisted methods of MBD quantification have been developed. The most common of these methods is a piece of software called Cumulus (University of Toronto, Toronto, Canada)(Byng, Boyd et al. 1994, Eng, Gallant et al. 2014), which uses semi-automated, user-interactive thresholding of the image to estimate the percentage of breast area that is dense tissue. The Cumulus method uses two-dimensional images of the breast; which has limitations because breast density is three-dimensional and potentially variable in appearance on two-dimensional mammograms due to differences in compression and the projection angle (Yaffe 2008). Also, different manufacturers of mammography machines have different imaging properties, which can alter the mean brightness of the image, in particular within areas that are difficult to penetrate such as fibroglandular tissue (Shaw, Albagli et al. 2004, Rivetti, Lanconelli et al. 2006), this difference can influence the measured MBD (Mahesh 2004, Shaw, Albagli et al. 2004, Rivetti, Lanconelli et al. 2006, McCullagh, Baldelli et al. 2011). Cumulus is also limited by intra- and inter-reader variability in establishing the threshold for segmenting dense tissue from the surrounding fatty tissue. The processes of measuring MBD with this method is labour intensive and time-consuming; this may be acceptable within research environments; however, the applicability of such methods in the real-world may be challenging (Jeffreys, Harvey et al. 2010).

1.2.2.3 Fully Automated Methods

The benefit of fully automated methods is that they decrease the subjectivity and operator dependency of the outcome measure. Four main fully automated methods have been validated: CumulusV™ (Sunnybrook Health Sciences Centre, Toronto, ON, Canada), Quantra™ (Hologic Inc, Bedford, MA, USA), VolparaDensity™ (Volpara Health Technologies, Wellington, New Zealand) and Densitas™ (Densitas Inc, Halifax, NS, Canada). These systems use images generated by digital mammography; algorithms calibrate pixels values in, depending on the system, the raw “for processing” or processed, full-field digital mammography images using a

model of X-ray physics and imaging parameters. These systems provide the outputs of volumetric per cent density (VPD) or as an absolute measure of dense and non-dense tissue (Highnam, Brady et al. 1996, Alonzo-Proulx, Mawdsley et al. 2015, Astley, Harkness et al. 2018). In the local setting of this research, VolparaDensity™ is the predominant software used and will be described in greater detail.

VolparaDensity™ is a volumetric breast density assessment software; it uses an algorithm to map the brightness across the mammogram. This brightness represents either the thickness of fibroglandular tissue or fatty tissue that is present between the pixel and the x-ray source. As the VolparaDensity™ algorithm runs across the image, it calculates the total fibroglandular volume (TFV); by using the compressed breast thickness and projected area, the total breast volume (TBV) is also able to be calculated. When the TFV is divided by the TBV, the percentage volumetric breast density (%VBD) is calculated. The breast density is provided per breast, which is obtained from averaging the values of the craniocaudal and mediolateral oblique images (Lee, Sohn et al. 2015).

Automated breast density analysis techniques rely on the brightness of the glandular and fatty tissue. For this reason, to date, there have been resulting difficulties with the consistency and reproducibility, as differences in the imaging parameters that change the brightness and contrasts of the image may affect the breast density calculations (Jeffreys, Harvey et al. 2010).

1.2.3 Factors that can Influence Mammographic Breast Density

1.2.3.1 Heritability and Mammographic Breast Density

Two studies have demonstrated that genetics may influence a woman's MBD (Boyd, Dite et al. 2002, Ursin, Lillie et al. 2009). Boyd, Dite et al. (2002) conducted a study on two samples of female monozygotic (identical) and dizygotic (non-identical) twins from Australia, Canada and The United States. After adjustments for age and other covariates, the combined correlation coefficient was 0.63 for monozygotic pairs and 0.27 for dizygotic pairs. Similarly, Ursin, Lillie et al. (2009), also studying monozygotic and dizygotic twins found that after adjustments for exposure and non-heritable risk factors that are known to influence MBD, genetics accounted for 53% in the variance for PMD and 59% for the variance in absolute breast density. Both of

these studies observing a greater correlation with MBD between members of monozygotic twins rather than dizygotic twins, alluding to potential genetic influence.

1.2.3.2 Parity Status and Number of Births

Numerous studies have linked MBD to parity and number of births. It has been demonstrated that parity is strongly correlated with MBD, with nulliparous (given birth to no children) women having a greater MBD compared to primiparous (given birth to one child) and multiparous women given (birth to multiple children) (de Waard, Rombach et al. 1984, Oza and Boyd 1993, Gram, Funkhouser et al. 1995, Hendriks, Otten et al. 2000, Li, Sun et al. 2005). It has also been shown that the number of births has a negative association with MBD (Li, Sun et al. 2005) with one study finding 47% of the nulliparous women had >25% density, compared to 37% of the women with one to three children and 19% of the women with more than three children (Hendriks, Otten et al. 2000). It is hypothesised that the strong relationship between parity and MBD is the reason parity has a protective effect regarding breast cancer risk (de Waard, Rombach et al. 1984).

1.2.3.3 Race and Ethnicity

There is conflicting evidence that race and ethnicity have an influence on MBD with the International Consortium on Mammographic Density (ICMD) (McCormack, Burton et al. 2016) being established to generate more evidence on this topic. One study by Heller, Hudson et al (2015) found that Chinese women were found to have significantly greater PMD and the lowest overall breast volume. Furthermore, the PMD of black females did not differ substantially from the cohort, having both a higher fibroglandular volume and also overall breast volume (Heller, Hudson et al. 2015).

McCormack, Perry et al. (2008) conducted a study comparing the MBD of Afro-Caribbean, South Asian and Caucasian women living in the United Kingdom. After adjusting for age, body mass index (BMI), menopausal status, use of hormone therapy, number of live births, age at first birth, family history of breast cancer and use of oral contraceptives. Afro-Caribbean and South Asian women had a lower mean MBD of -1.3% (95% confidence interval (CI) -3.7% to 1.3%) and -3.8% (95% CI -6.3% to 1.1%) compared to Caucasians, respectively. In regard to the Afro-Caribbean women, 60% of the differences in MBD were attributed to a higher mean

BMI, earlier menopause, lower prevalence of hormone therapy and a greater number of live births. As for the South Asian women one third of the reduced density was explained by BMI and reproductive characteristics, but the fully adjusted differences remained statistically significant.

Mariapun, Li et al. (2015) examined MBD in Chinese, Malay and Indian women and found that Chinese women had significantly higher PMD compared to Malay and Indian women after adjustment for age, BMI, menopausal status, parity and age at first full term pregnancy. The difference in MBD between the ethnicities predominately coming from the lower non-dense area in Chinese women.

Del Carmen, Halpern et al. (2007) reported that MBD amongst Asian women was significantly greater compared to Caucasian, African American, Indian and Caribbean or non-disclosed races. However, within this study in all the other groups, breast density did not correlate with race beyond what can be attributed to differences in BMI, bra size and cup size. Maskarinec, Nagata et al. (2002) compared the MBD of three groups of women, these being white women, Japanese women living in Hawaii, and Japanese women living in Japan; the findings showing that PMD was higher in Japanese women in Hawaii compared to those living in Japan. The conclusion was that the size of the TBV differs primarily by race and the proportion of dense area by place of residence (Maskarinec, Nagata et al. 2002).

The evidence currently shows that race and ethnicity may be an influencing factor for MBD; however other environmental factors and exposures, such as where the woman is living, may have been more influential for MBD differences. Other unexamined factors that may explain ethnic differences include dietary intake, in particular with calcium, vitamin D, fat, and phytoestrogens, which in some studies have been related to MBD (Knight, Martin et al. 1999, Vachon, Kushi et al. 2000, Bérubé, Diorio et al. 2004, Maskarinec, Takata et al. 2004). In addition, known lifestyle determinants of MBD do not fully account for the ethnic variations in MBD, in particular within Asian cohorts.

1.2.3.4 Age and Mammographic Breast Density

It is known that hormonal changes that occur during menopause lead to alterations of the glandular features of the breast tissue. Two studies have examined the relationship between

age and MBD (Li, Sun et al. 2005, Checka, Chun et al. 2012) and both found an inverse relationship between these two variables. Checka, Chun et al. (2012) found that 74% of patients between 40 and 49 years of age had dense breasts, this decreasing to 57% of women in their 50's, 44% of women in their 60s and 36% of women in their 70's. Within these results, there were exceptions across all age groups, and there was similar variability within each age group. These studies demonstrate that age may not be a perfect surrogate for breast density but remains an influencing factor.

A further study by Burton, Maskarinec et al. (2017) examined differences in MBD, concerning the age and menopausal status, from data collected from women from 22 countries. It was found that regardless of ethnicity or the country the women were from, MBD was much lower in postmenopausal compared to premenopausal women of the same age. In addition to this, proportionally to the breast area, the breast density was lower in older women both among premenopausal and postmenopausal women. In premenopausal women, MBD changed with age without an increase in the breast area; however, the latter (breast area) also increased amongst postmenopausal women. These findings demonstrate that within a population of women from multiple countries, there is a consistent effect of age and menopausal state on MBD, suggesting that these associations may reflect a common biological process. At the tissue level, changes which involve the area of high density arise from changes in the stromal tissue or in the epithelial tissue or both stromal tissue and epithelial tissue. At the same time, the changes in the PMD are also affected by changes in the adipose tissue within the breast. Areas of decreasing density likely reflect involution of terminal duct lobular units (TDLUs). X-rays of histological tissues showed that TDLUs have raised concentrations of radiologically dense areas and that these areas also declined with age (Gierach, Patel et al. 2016). The age-related decline in MBD may, however, be modified by external factors such as hormonal or reproductive factors or lifestyle or environmental factors.

1.2.3.5 Use of Hormonal Therapies and Mammographic Breast Density

Hormonal therapies and natural hormonal fluctuations that occur throughout a woman's life are known to influence and have the ability to modify MBD. Even the fluctuations during a woman's menstrual cycle can significantly alter MBD (Ursin, Parisky et al. 2001). As mentioned in Section 1.2.3.4, MBD decreases throughout menopause in response to changes

that occur in a woman's hormones. Combination HRT has consistently been shown to increase MBD (Persson, Thurfjell et al. 1997, Greendale, Reboussin et al. 1999, Lundström, Wilczek et al. 1999, McTiernan, Martin et al. 2005, (Chlebowski, Hendrix et al. 2003), specifically oestrogen with progestin therapy, which has been found to increase MBD five to seven times more than oestrogen alone, which had a small or negligible effect (Greendale, Reboussin et al. 1999). In addition to this, tamoxifen, which is a partial antiestrogenic therapy, has consistently shown to decrease MBD (Atkinson, Warren et al. 1999, Brisson, Brisson et al. 2000, Chow, Venzon et al. 2000, Konez, Goyal et al. 2001, Cuzick, Warwick et al. 2004).

1.2.4 Current Interventions for Reducing Mammographic Breast Density

1.2.4.1 Hormonal Interventions

As mentioned in Section 1.2.3.5, MBD can be influenced in response to changes in hormonal exposures. Androgens and oestrogens have competing actions within normal breast tissue, and the regulation of oestrone (E_1), oestrone-sulphate and oestradiol (E_2) levels rather than the concentration of oestrogen in the breast tissue may be associated with changes in MBD (Vachon, Suman et al. 2013). As the breast is embryologically a modified sweat gland, it responds like a sweat gland to any alteration to its oestrogen and androgen (E/A) ratio (McNally and Stein 2017). When there is a shift in the E/A ratio towards an androgenic tissue environment in the breast, there is a substantial change in MBD (as is seen following menopause (Boyd, Martin et al. 2002)). Therefore, to reduce MBD, there needs to be a profound impact on oestrogenic action, which is difficult in premenopausal women without imposing undue systematic effects; therefore, outweighing the benefit of therapy. To date, two classes of pharmaceutical interventions have shown to be efficacious at reducing MBD; these are selective oestrogen receptor modulators (SERMs) and aromatase inhibitors (Ais). In addition, non-steroidal anti-inflammatory drugs (NSAIDs) and the antidiabetic agent metformin have also been evaluated for effect on MBD reduction.

1.2.4.2 Selective Oestrogen Receptor Modulators (SERMS)

Two SERMs have been examined for the use of reducing MBD in both premenopausal and postmenopausal women; these are tamoxifen and raloxifene. Selective oestrogen receptor modulators exert agonist or antagonist effects on various oestrogen target tissues (Riggs and Hartmann 2003). Shang and Brown (2002) found that tamoxifen and raloxifene act as anti-

oestrogens within the breast, and as partial agonists of oestrogen, they can cause significant alterations in both breasts and systemic oestrogenic stimulation. This change in systemic oestrogenic stimulation causes a shift in the E/A ratio, creating a more androgenic environment and reducing cellular proliferation in the breast, thus having the ability to reduce MBD.

There have been ten studies which have assessed the use of tamoxifen for MBD, seven of these including both pre-and postmenopausal women. Within these ten studies, all of them reported tamoxifen mediated MBD decreases. Nine studies assessed the efficacy of raloxifene on MBD, with eight of these studies including only postmenopausal women. Of these nine studies, only three reported a statistically significant reduction in MBD. A summary of the results for these studies on tamoxifen and raloxifene are reported in Table 1-1.

Table 1-1: Summary table of studies utilising SERMs to influence MBD

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Brisson, Brisson et al. (2000)	RCT	≥ 35 years Cases (n) = 36 Controls (n) = 33	Tamoxifen 20mg/day for 5 years	PMD and Wolfes Parenchymal Pattern	Mean PMD reductions: 1.0 – 3.4 years = -6.9% ± 11.1%; 3.5 – 5 years = -10.9% ± 12.4%. Overall PMD Reductions: Tamoxifen = -9.4%, Placebo -3.6% P= <0.01.
Cuzick, Warwick et al. (2011)	Nested CCS	30 – 70 years Cases (n) = 123 Controls (n) = 942	Tamoxifen 20mg/day for 5 years	PMD (Boyd's SCC, Cumulus)	Compared to all women in the placebo group, women in the tamoxifen group who experienced 10% or greater reduction in breast density had 63% reduction in BC risk: OR = 0.37, (95% CI 0.20 to 0.69, P=0.002).
Cuzick, Warwick et al. (2004)	Nested CCS	35-75 Years Cases (n) = 388 Controls (n) = 430	Tamoxifen 20mg/day for 5 years	PMD (Boyd's method, visual assessment)	BD reductions: 18 month follow up -7.9% (95% CI 6.9% to 8.9%; p=0.001). 54 month follow up -13.7% (95% CI 12.3% to 15.1%; p<0.001). There were reductions in the placebo group, but the reductions were greater in the tamoxifen group than the placebo group (p=0.001).
Chen, Chang et al. (2011)	CS	33-51 Years Cases (n) = 16	Tamoxifen 20mg/day for 8-26 months	%FV, PMD (MRI: computer-assisted algorithm)	BD reductions after 17 months = -5.8% ± 3.8% (p<0.001). 7 subjects showed absolute reduction of %BD less than -5%; 7 were between -5-10%; 2 larger than -10%.
Decensi, Robertson et al. (2009)	RCT (*post-hoc* analysis)	Premenopausal BC women (n) = 235	Tamoxifen 5mg/day for 2 years	PMD (Boyd's and Cumulus)	MBD; Month 12 (Boyd and Cumulus) = -9.9% (95% CI -16.2 to -3.6). Month 24 (Boyd and Cumulus) = -16.2 (95% CI -22.6 to -9.8) Month 12 (analogue only) -13.8 (95% CI -20.0 to -7.6); Month 24 = -19.6 (95% CI -26.3 to -12.9).

Table 1-1 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Atkinson, Warren et al. (1999)	RCT (*post-hoc* analysis)	50-64 years Cases (n) = 94 Controls (n) = 188	Tamoxifen 20mg/day for 2 years	PMD (Wolfe's Method)	Significant change toward a less dense pattern in the case group (p = 0.0001) after treatment with tamoxifen. The change was significant in the case group compared to the control group (p=0.0001). The OR associated with the denser P2 and DY patterns vs the less dense N1 and P1 patterns was 3.6 (95% CI 2.11 to 6.18) at the initial mammogram. This was significantly reduced to 1.5 (95% CI 1.32 to 1.70) by the follow-up mammogram (p=0.019, X^2 = 5.52 for the difference between the two ORs).
Chow, Venzon et al. (2000)	RCT (*post-hoc* analysis)	36-74 years High risk BC women (n) = 32	Tamoxifen 20mg/day for 23 months	PMD (Wolfe's, BI-RADs, Boyd's SCC)	Using the first and last digitised scores, 56% of participants showed a relative decrease of ≥10% mean, -10%; SD 16%). The digitised breast density changes were -4.3%, SD 6.60%, range -21.5 to 10.1%, P=0.0007. All three other criteria showed some decreases (Wolfe average -0.03, SD 0.4, range -1.0 to 1.5, p=0.05; BI-RADs average -0.1, SD 0.4, range -1.5 to 0.05; p=0.12; semi-quantitative average -0.2, SD 05, range -1.2 to 0.8, p=0.039). Only semi-quantitative scores achieved statistical significance.

Table 1-1 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Konez, Goyal et al. (2001)	RCT (*post-hoc* analysis)	32-81 years unaffected breasts of women following surgery for unilateral BC (n) = 27	Tamoxifen 20mg/day for 5 years	PMD (Visual assessment)	19 cases (79%) showed no categorical change during or after the course of treatment (17 of the 19 showed fatty or minimally dense breast parenchyma). 3 (13%) showed a reduction in density within the first half of the treatment. All density changes were only by one level (e.g. minimally dense to fatty). Densitometer readings demonstrated a minimal reduction in glandular density during tamoxifen treatment in 14 cases (52%) within the first half and in 16 cases (60%) at the end of the course of treatment. Within the first half, only 4 cases showed more than 10% reduction in glandular density, which was noted in 7 additional cases at the end of the course of treatment.
Meggiorini, Labi et al. (2008)	RCT (*post-hoc* analysis)	Mean age 58.5 ± 9.3 years Cases (n) = 68 Controls (n) = 80	Tamoxifen 20mg/day for 1 year	PMD (BI-RADs, Cumulus)	Significant decrease in density ($p<0.005$).
Son and Oh (1999)	RCT (*post-hoc* analysis)	28-67 years 152 patients with BD, Healthy women (n) = 20 Tamoxifen (n) = 102 Control (n) = 70	Tamoxifen 20mg/day for 2 years	Visual Assessment	On follow up mammograms, 61 (59.8%) of 102 patients in the tamoxifen group showed a decrease in breast parenchymal area, 59 (57.8%) had more clearly visualised Cooper's ligament, and 59 (57.8%) had clearly visualised ducts. A decrease in parenchyma was seen in 18 (36%) of 50 patients in the non-tamoxifen group and two (10%) of the 20 healthy women. This was a significant decrease in density ($p<0.005$).

Table 1-1 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Cirpan, Akercan et al. (2006)	RCT (retro-analysis)	Postmenopausal n = 55	Raloxifene 60mg/day for 16 months	PMD (BI-RADs)	Null.
Eng-Wong, Orzano-Birgani et al. (2008)	Phase II open-label	Premenopausal – 35-47 years Phase II trial of raloxifene (n) = 27	Raloxifene 60mg/day for 2 years	PMD; MRIV (thresholding technique)	No significant change, mean change at 1 year was 1% (95% CI -3 to 5), 2 years 1% (95% CI -2 to 5). MRIV decreased on raloxifene – median relative change after 1 year was -17% (95% CI -28 to -9; p=0.0017), after 2 years -16% (95% CI -31 to -4; p=0.0004).
Freedman, Martin et al. (2001)	RCT	45- 60 years n = 168 Placebo (n) = 45 60mg/day raloxifene (n) = 45 150mg/day raloxifene (n) = 42 oestrogen (n) = 36	Raloxifene 60mg/day or 150mg/day for 2 years oestrogen 0.625mg/day	PMD (computer-assisted techniques)	Results at 2 years: placebo and both raloxifene groups had PMD decreases. Placebo mean change = -1.3% (95% -2.2% to -0.4%;p=0.003) Raloxifene 60mg/day mean change = -1.5% (95% CI -2.7% to -0.3%; p=0.002). Raloxifene 150mg/day mean change = -1.7% (95% CI -2.8% to -0.6%; p<0.001). Oestrogen mean change = 1.2% (95% CI -0.6% to 3.0%; p=0.611)

Table 1-1 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Harvey, Holm et al. (2009)	retrospective subset of women enrolled in an RCT	≤62 years Raloxifene (n) = 119 Placebo (n) = 125 Bazedoxifen 20mg 0r 40mg (n) = 92, 106	Raloxifene 60mg/day for 2 years	PMD (cumulus)	Results after 2 years, mean per cent change in PMD was Raloxifene -0.5% (95% CI -1.1 to 0.1); Placebo -0.2% (95% CI -0.7 to 0.4). ANOVA showed no significant differences between the groups.
Harvey, Pinkerton et al. (2013)	Ancillary study of phase III RCT	55.2 to 56.3 years n = 507 postmenopausal non-hysterectomised women	Raloxifene 60mg/day for 2 years	PMD (Cumulus)	Mean PMD change in the raloxifene group was -0.23%; (95% CI -0.54% to 0.08%); placebo mean change was -0.42 (95% CI -0.72 to -0.11). There were no significant differences between the group.
Jackson, San Martin et al. (2003)	RCT	≥60 years – Raloxifene 66.9 ± 5.3; CCHT 66.4 ± 4.5 Raloxifene (n) = 84 CCHT 9n) = 109	Raloxifene 60mg/day for 1 year	Mean BD (BI-RADs) Non-BI-RAD visual radiology assessment (increased, unchanged, decreased, unevaluable)	From baseline to 12 months, 0.9% of the women in the raloxifene group had an increase in breast density, and 99.1% showed no change. In the CCHT group, 70.2% had an increase, and 29.8% showed no changes. There was a statistically significant difference between the treatment groups p<0.001.

Table 1-1 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Lasco, Gaudio et al. (2006)	CCS	Postmenopausal Women with mean BMI 24.7, 52.4 ± 4.1 years n = 70	Raloxifene 60mg/day orally for 2 years	Image Pro-Plus as hoc software Image Mean Index (IMI)	Null – after 24 months of therapy, in the raloxifene group, there was a significant variation in mammary density compared with baseline. No significant variations were observed. The IMI value decreased significantly only in the raloxifene-treated women (-1.9%) $p<0.05$. IMI decreased but not significantly in the control group.
Nielsen, Raundahl et al. (2009)	RCT (retro analysis)	55-80 years n = 135	Raloxifene 60mg/day for 2 years or transdermal (E2)	PMD (BI-RADs, computer analysis)	Null – BI-RAD score did not increase significantly in either treatment group, and the treatment effects were not significantly different between the two treatment groups. The area percentage increased in both groups and significantly in the E2 group, which was significantly more than in the raloxifene group.
Silverio, Nahas-Neto et al. (2007)	RCT	61.1 years mean n = 80	Raloxifene 60mg/day for 2 years	PMD (BI-RADs and computer-assisted)	Null – after 6 months, no alteration was observed in the MBD in 38 women of the raloxifene group and 38 of the control group by a qualitative method. By a quantitative method, no alteration was observed in 30 women of the raloxifene group and 27 controls ($p>0.05$)
Christodoulakos, Lambrinoudaki et al. (2002)	Cohort Prospective Study	41-67 years n = 131	Tibolone 2.5mg/day and raloxifene 60mg/day for 1 year	PMD (Wolfe's Method)	Null – 10.7% of women in the tibolone group showed an increase in breast density and 6.3% showed an increase in the raloxifene group. No women in the control group showed an increase in breast density. Between-group differences did not reach statistical significance.

Table 1-1 continued

Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results	
Eilertsen, Karssemeijer et al. (2008)	RCT	45-65 years n = 177	Raloxifene 60mg/day or tibolone 2.5mg/day 12 weeks	VBD (automated physics-based method)	Median reduction -4.1% (95%CI -6.9 to 2.1%) in raloxifene group Median reduction of 0.7% (95% CI -9.2 to 7.3%) in the tibolone group, both changes were statistically insignificant.

%FV - per cent fibroglandular volume
BC – breast cancer
BD – breast density
CCHT – continuous-combined hormone therapy
CCS – case-control study
CS – cohort study
MRI – magnetic resonance imaging
MRIV – magnetic resonance imaging volume
PMD – per cent mammographic density
RCT – randomised controlled trial
SCC – six class categories
SD – standard deviation
VBD – volumetric breast density

Although tamoxifen has been shown to be efficacious at reducing MBD, as tamoxifen causes both a shift in breast and systemic oestrogen stimulation, it has been associated with significant adverse events (AEs), such as increased risk of ischemic stroke, uterine/endometrial cancers, vaginal discharge and hot flushes (Oncology 2009), which may outweigh the benefit on the therapy and reduce compliance with research stating that only 4% of women at increased risk of breast cancer have accepted the use of tamoxifen as a chemopreventative therapy (Ropka, Keim et al. 2010).

1.2.4.3 Aromatase Inhibitors

Another class of pharmaceutical intervention that has been found to reduce MBD are aromatase inhibitors. Aromatase is an enzyme of the cytochrome P-450 superfamily and the product of the CYP19 gene and is expressed in several tissues including subcutaneous fat, liver, muscle, brain, and normal breast tissue (Nelson and Bulun 2001). The aromatase enzyme is responsible for the conversion of the adrenal androgen substrate androstenedione to oestrogen in peripheral tissues (Evans, Ledesma et al. 1986), with peripheral tissue being the predominant source of oestrogen in postmenopausal women (Altundag and Ibrahim 2006). As the E/A ratio needs to be shifted to an androgen environment to benefit MBD; Ais reduce oestrogenic drive by blocking the conversion of androstenedione to E_1 and testosterone to E_2 decreasing both serum and breast tissue oestrogen levels (Dowsett, Jones et al. 1995, Miller and Dixon 2001, Geisler, Haynes et al. 2002, Ingle, Buzdar et al. 2010). Aromatase inhibitors are contraindicated in the treatment of breast cancer of premenopausal women; the rationale for this assertion is that in premenopausal women, oestrogen is predominately produced in the ovaries unlike postmenopausal women, where it is predominately produced in peripheral tissues by aromatisation of androgens. If Ais are used as a sole treatment in premenopausal patients, it leads to interference with the negative feedback mechanism between the ovaries and the pituitary gland, which can result in hypothalamic-pituitary inhibition, stimulating the release of Gonadotropin-releasing hormone (GnRH), leading to ovarian hyperstimulation (Mitwally and Casper 2001, Casper 2007). This ovarian hyper-stimulation being the reason that Ais are used for infertility treatment to induce ovarian hyper-stimulation for follicular genesis.

A total of nine studies have studied the effect of Ais on MBD. Only one, an open label, reported a statistically significant reduction in MBD. The other two studies with reductions in

MBD had no significant changes from the placebo or control treatments or control participants. A summary of the Ai findings is reported in Table 1-2.

Table 1-2: Summary table of studies utilising AIs to influence MBD

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Cigler, Richardson et al. (2011)	RCT (placebo-controlled)	Healthy postmenopausal women with measurable MBD 50 years or greater Exemestane (n) = 49 Placebo (n) = 49	Exemestane 25mg/day for 12 months	PMD (computer assisted method)	6 month follow up: Mean absolute change in PMD from baseline. Exemestane = -1.33 (95% CI -5.55 to 2.89; p=0.53); Placebo = 0.22 (95% CI -4.59 to 5.02; p=0.93). 12 Month follow up: Mean absolute change in PMD from baseline Exemestane = 0.56 (95% CI -3.98 to 5.11; p= 0.80); Placebo = 0.58 (95% CI -4.69 to 5.86; p = 0.82). 24 Month follow up: mean absolute change in PMD from baseline Exemestane = -0.17 (95% CI -4.34 to 4.00; p=0.93); Placebo = -2.93 (95% CI -8.70 to 2.85; p = 0.30) the change at 12 months and 24 months did not differ significantly between the two groups.
Cigler, Tu et al. (2010)	RCT (placebo-controlled)	Postmenopausal women with or without a history of early-stage breast cancer Baseline mammogram demonstrating density occupying greater than 25% of the breast Letrozole (n) = 44 Placebo (n) = 23	Letrozole 2.5mg/day for 12 months	PMD (computer-assisted method)	The mean (absolute) change in PD from baseline at 12 months was -1.74% (95% CI -3.85% to 0.37%; p= 0.10) for the letrozole group; placebo was -0.24% (95% CI -4.47% to 4.26%). There was no statistically significant change between the two groups. The mean (absolute) change in PMD from baseline and 24 months was -0.01% (95% CI -3.89% to 3.87%; p = 0.99) for women on letrozole and -1.32% (95% CI -8.86% to 6.22%; p = 0.71) for women on placebo. There was no statistically significant difference between the two groups (p=0.69) and after adjustment for age and BMI (p=0.61).

Table 1-2 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Henry, Chan et al. (2013)	RCT	Postmenopausal women with stage 0-III hormone receptor positive breast cancer Letrozole (n) = 139 Exemestane (n) = 120	Exemestane 25mg/day for 2 years or letrozole 2.5mg/day for 2 years	PMD (BIRADs, MDEST)	There was a statistically significant absolute mean decrease in PMD of -1.9% (SD 4.9%), which corresponded to an average percentage decrease of -5.6% (SD 34.7%). The baseline PMD of those subjects whose PMD decreased with Ai therapy was 19.4% (SD 10.9%), whereas the baseline PMD of those subjects whose MPD was stable or increased was 12.6% (SD 8.8%). For those subjects with baseline MPD ≥20%, the average absolute decrease in MPD with 2 years of Ai therapy was -4.7% (SD 5.5%), whereas for those subjects with baseline PMD <20%, the average absolute decrease in PMD was -0.6% (SD 4.0%). This difference was statistically significant (P=0.00001) The absolute change did not differ between the treatment groups.
Prowell, Blackford et al. (2011)	RCT	Postmenopausal women >60 years with histologically confirmed hormone receptor-positive DCIS or stage I-III invasive breast cancer	Anastrozole 1mg/day for 1 year	PMD (cumulus) of contralateral breast	At 12 months compared to baseline, there was a non-statistically significant reduction in PMD -16% (95% CI -30% to 2%, p=0.08).
Smith, Dilawari et al. (2012)	Pilot trial – Open-Label trial	Postmenopausal women 50 years or greater Participants also needed to meet one or more criteria for increased risk of BC Letrozole (n) = 16	Letrozole 2.5mg/day for 12 months	PMD (Madena Software) – total dense area as well as percentage density	At 6 months, 8 women had already shown a decrease in PMD, whereas, at 12 months, eleven had a decrease in PMD relative to that at baseline. 3 women exhibited an overall absolute increase in PMD during the conduct of the study, including two who had decreased at 6 months. The overall difference between baseline and 12-month densities was statistically significant (two-tailed p=0.0358) on a two-sided t-test comparison.

Table 1-2 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Fabian, Kimler et al. (2007)	Open-Label Study	Postmenopausal women with increased risk of breast cancer 42 subjects median age 50 (range 39-68)	Letrozole 2.5mg/day for 6 months	PMD (Cumulus) – per cent dense area compared to the entire breast area	There were no significant changes in PMD.
Mousa, Crystal et al. (2008)	Retrospective cohort study	Postmenopausal women (n) = 28	Low-dose HT plus letrozole 2.5mg 3/per week Controls just using HT alone	PMD (BI-RADs and ImageQuant)	ImageQuant showed a statistically significant reduction in PMD in women taking Ai plus HT. There were no significant changes in the control group. The BI-RAD system was less sensitive to change.
Vachon, Ingle et al. (2007)	Pilot RCT study	NCIC CTG MA-17 Study population n= 104 Letrozole (n) = 56 (54%) Placebo (n) = 48 (46%)	Letrozole 2.5mg/day for 1 year	PMD (cumulus)	The change in PMD at 1-year post-randomisation was not found to differ between the letrozole group (unadjusted mean -0.8%) and placebo group (unadjusted mean -0.6%) (p=0.76). No difference between the treatment groups was found in terms of change in PMD after adjusting for age, BMI, nodal status, number of tumours, and time on tamoxifen. Longitudinal changes in PMD across time were similar in the letrozole groups, unadjusted mean -0.69 (95% CI -1.33 to -0.06) and adjusted group -0.68 (-1.34 to -0.02), p=0.24 and the placebo groups, unadjusted mean -0.18 (-0.84 to 0.49) and adjusted group -0.12 (-0.84 to 0.59), p=0.23.

Table 1-2 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Vachon, Suman et al. (2013)	CCS	Postmenopausal women with early-stage breast cancer n = 387 (369 had matched controls)	Anastrozole 1mg/day or Exemestane 25mg/day for 1 year	PMD (Cumulus)	The median difference for all 369 pairs was 0.1% (with 10th percentile of 5.9% and 90th percentile of 5.2%). Thus, there was no evidence to conclude that the change in PMD over 1 year differs between a case and her matched control whether considering all matched pairs (n=369; p = 0.51). Of the 387 postmenopausal breast cancer women receiving an average of 10 months of adjuvant Ai therapy, 15% experienced at least a 5% decrease in their MBD. This increase to 20% for the 280 cases who had a baseline PMD of at least 10%. Multivariate analyses showed the likelihood of experiencing a reduction of at least 5% in PMD with Ai therapy was increased for cases with a baseline density of 15% or more (OR 10.4; 95% CI 4.0 to 26.9; p<0.0001) who had 12 months or more of Ai treatment (OR 3.18; 95% CI 1.68 to 6.03; p = 0.0004). The median difference of all 369 pairs was -0.1% (with 10th percentile of -5.9% and 90th percentile of 5.2%). No evidence that the change in PMD over 1 year differs between a case and her matched controls (n=369; p=0.51).

ADH – atypical ductal hyperplasia
Ai – aromatase inhibitor
ALH – atypical lobular hyperplasia
BC – breast cancer
CCS – case control study
CI – confidence interval
DCIS – ductal carcinoma in situ
HT – hormone therapy
LCIS – lobular carcinoma in situ
MDEST – mammographic density estimator
MPD – mammographic per cent density
PD – per cent density
RCT – randomised controlled trial

1.2.4.4 Non-steroidal Anti-inflammatory Drugs

Another class of pharmaceutical intervention that has been examined for its influence on MBD are non-steroidal anti-inflammatory drugs (NSAIDs), with five studies having been completed analysing their influence on MBD. This class of drug works by inhibiting cyclooxygenase, which is an enzyme responsible for catalysing the synthesis of prostaglandins. Prostaglandins have been shown to increase aromatase gene expression and thereby also increase oestrogen production (Zhao, Agarwal et al. 1996) as well as stimulate progesterone synthesis (Elvin, Yan et al. 2000); with both oestrogen and progesterone driving cell proliferation. This increase in cell proliferation can lead to an increase in MBD. As NSAIDs inhibit cyclooxygenase, this results in a negative feedback loop, which is hypothesised to decrease oestrogen and progesterone synthesis; with this having the potential to result in favourable changes to a woman's MBD. The summary of each of the five studies is reported in Table 1-3.

Table 1-3: Summary of studies utilising NSAIDs to influence MBD

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Wood, Sprague et al. (2017)	Retrospective cohort study	26,000 women included in the study mean age 57.3 (range 40-89) 19.6% were under the age of 50 13.9% were over the age of 70	Current Aspirin Use No Aspirin Use Use <300 mg/day Use >300mg/day Between 2012 - 2013	BI-RADs	A greater proportion of aspirin users had BI-RADs 1 and 2 densities than non-users (72.8 vs 54.3%; p<0.001). After adjusting for age, BMI, and ethnicity, there was an independent, inverse association between aspirin use and MBD (P trend <0.001). Compared with women with scattered fibroglandular tissue, women with either heterogeneously (OR 0.84; 95% CI 0.78 – 0.92) or extremely dense (OR 0.73; 95% CI 0.57 – 0.93) breast were less likely to be aspirin users, while women with entirely fat breasts were more likely to use aspirin (OR 1.15; 95% CI 1.04 – 1.27). Women with dense breasts were less likely to be aspirin users than those with non-dense breasts (OR 0.82; 95% CI 0.76 – 0.89), this effect was also seen when density was analysed as a dichotomised variable (dense = BI-RAD 3 to 4 and non-dense BI-RAD 1 to 2). A lower likelihood of having dense breasts (BI-RADs 3+4) with increasing aspirin dose (OR 0.62; 95% CI 0.50 – 0.76) for >300mg compared to non-users; P trend 0.007.

Table 1-3 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Stone, Willenberg et al. (2012)	Retrospective cohort study	Participants were part of two national studies in Australia Female twin pairs aged 40-70 years Sister pairs aged 40-70 years affected by endometriosis 3286 Participants Average age 54.5 (SD 8.4) 69.2% postmenopausal	Observed NSAID use for a time period of 0-5 years	PMD (cumulus)	After adjusting for covariates, there was no evidence of associations between square root PMD and any NSAIDs use, frequency, and duration (all P trend >0.2). After adjusting for all the necessary covariate, there was no evidence of an association between square root PMD and all the respective NSAIDs (all P trend >0.06). There was no difference in the interpretation of the separate estimates for pre- or postmenopausal.
McTiernan, Wang et al. (2009)	RCT (placebo-controlled)	Postmenopausal aged 50 to 75 years Not using menopausal hormone therapy, oral contraceptives or SERMs for the previous 6 months Aspirin group (n) = 75 Placebo Group (n) = 68	Aspirin 325mg/day Placebo identical appearing placebo capsule for 6 months	PMD (cumulus)	PMD decreased in women randomized to aspirin by an absolute -0.8% versus an absolute decrease of -1.2% in controls (P=0.84). There was no statistically significant difference between the two trial arms. No observed effect of aspirin on PMD. Aspirin also did not affect density differently than placebo when we looked at subgroups of women characterized by age, BMI, or baseline PMD.

Table 1-3 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Maskarinec, Urano et al. (2008)	Cross-sectional data analysis with data from two cohort trials	Multi-ethnic cohort Postmenopausal and premenopausal women 218 from the BEAN study and 1247 from the NCC study Mean age from BEAN study was 43.0 ± 2.8 years and 58.7 ± 8.6 years for the NCC subject Mean per cent density was 46.9% in the BEAN study, whereas it was 32.5% in the NCC study	NSAID use	PMD (computer-assisted)	In the combined study population, no statistically significant association was observed between any medication use and mean PMD Current analysis did not show a statistically significant association between NSAID use and PMD in this multi-ethnic study population Women with long-term NSAID use had non-significantly higher PMD than non-users although short-term users had slightly lower PMD than non-users Results differed by menopausal status; the trend of higher PMD with a longer duration of NSAIDs use was significant among postmenopausal women, PMD was slightly lower among premenopausal women with long term NSAID use.

Table 1-3 continued

Author, Year	Study Design	Study Population (age and n)	Type of Intervention	Outcome Measures	Results
Terry, Buist et al. (2008)	Retrospective cohort	Women who had two routine screening mammograms within 9 to 28 months of each other between 1996 and 2006 Women were excluded if they reported ever using raloxifene or tamoxifen, women with a history of breast cancer or women who had breast augmentation or reduction n = 29,284	NSAIDs Continuers – women who had pharmacy dispensing for the entire period between the two mammograms Discontinuers were women who were dispensed a given class of NSAID at the first mammogram but had no dispensing within 6 months before the second mammogram Non-users did not have any pharmacy	Bi-RADS	Non-users of NSAIDs were more likely to be younger, have lower BMI, be never users of HRT and have dense breast tissue (BI-RADs category 3 or 4) Initiators and continuers of any NSAIDs were more likely to stay not dense than stay dense (multivariable OR 1.12; 95% CI 1.04 to 1.20; multivariable OR 1.25; 95% CI 1.05 to1.49, respectively) Point estimates were the same for initiators of non-prescription and prescription NSAIDs for staying not dense compared with staying dense (multivariable OR 1.11; 95% CI 1.03 – 1.20; multivariable OR 1.11; 95% CI 0.97 to 1.26, respectively) Discontinuers of non-prescription NSAIDs were more likely to decrease density compared with non-users (OR 1.40; 95% CI 1.10 to 1.79) compared with staying dense. There was no association with density change from discontinuation of prescription NSAIDs nor was there an association between continuation of NSAIDs (either prescription or non-prescription) and density change (increase or decrease) Continuers of non-prescription NSAIDs were more likely to stay not dense compared with those who stayed dense (OR 1.40; 95% CI 1.13 to 1.72)

Table 1-3 continued

			fills for a given class of NSAID within the 6 months before the second mammogram		Mixed users of non-prescription NSAIDs were more likely to increase density, whereas mixed users of prescription NSAIDs were less likely to increase density (OR 1.64; 95% CI 1.08 to 2.48; OR 0.58; 95% CI 0.35 to 0.96, respectively) Findings show that initiators and continuers of any NSAID were more likely to stay not dense compared with staying dense was limited to women aged <65 years (OR 1.24; 95% CI 1.12-1.36, OR 1.48; 95% CI 1.14 -1.93 for women ages <65 years compared with OR 1.02; 95% CI 0.92 -1.14; OR 1.18; 95% CI 0.93-1.48 for women aged 65 or greater. Overall, there was no reduction in MBD from initiation of NSAIDs by class or type of NSAID. There was also no observation that on the increase in MBD from discontinuation of use

CI – confidence interval
HRT – hormone replacement therapy
MBD – mammographic breast density
NSAIDs – non-steroidal anti-inflammatory drugs
OR – odds ratio
PMD – per cent mammographic density
RCT – randomised controlled trial
SERM – selective oestrogen receptor modulator

The results from the five studies have inconsistent findings. Three of the studies (Maskarinec, Urano et al. 2008, McTiernan, Wang et al. 2009, Stone, Willenberg et al. 2012) show no association between NSAIDs and MBD, and two studies demonstrate an inverse association with NSAIDs and MBD, which may reflect that age and demographics of users of NSAIDs, for example non users are younger and have a lower BMI. To date, none of the studies show significant evidence that NSAIDs can be used as an intervention for reducing MBD.

1.2.4.5 Metformin and Mammographic Breast Density

Metformin is commonly prescribed as an oral antidiabetic to patients with type two diabetes, and its action is to target the enzyme 5' adenosine monophosphate-activated protein kinase (AMPK). This enzyme induces muscles to take up glucose from the blood and increased the body's insulin sensitivity and improves glycaemic control (Evans, Donnelly et al. 2005, Col, Ochs et al. 2012).

Metformin has been found to reduce breast cancer risk (Col, Ochs et al. 2012) and It has been hypothesised that a possible pathway for this diabetic treatment and breast cancer risk reduction could be via an intermediary effect on MBD. Six studies have examined the association between diabetes and MBD (Roubidoux, Kaur et al. 2003, Sellers, Jensen et al. 2007, Tehranifar, Reynolds et al. 2014, Sanderson, O'Hara et al. 2015, Buschard, Thomassen et al. 2017).

In four of the studies, the diabetic women were found to have lower PMD compared with the non-diabetic women (Sellers, Jensen et al. 2007, Tehranifar, Reynolds et al. 2014, Sanderson, O'Hara et al. 2015, Buschard, Thomassen et al. 2017). One of the studies found a statistically significant inverse association between diabetes and MBD in the premenopausal participants but not in the postmenopausal women (Roubidoux, Kaur et al. 2003), however this study also found that increasing weight also lowered the odds of high MBD. Buschard, Thomassen et al. (2017) discovered an inverse association between MBD and diabetes for women who controlled their condition with diet or antidiabetic agents, while women taking insulin showed a positive association with having mixed/dense breasts; however, this finding was not statistically significant. Oskar, Engmann et al. (2018) also found that in women with type 2 diabetes who used metformin, there was an associated average 5.7% (95% CI -10.27 to -1.19)

lower PMD as compared with the group without type 2 diabetes, this association was significantly attenuated after adjusting for BMI.

From these six studies, it can be seen that there is a trend for women with diabetes to have a lower MBD when compared to women without diabetes. However, this result may have been confounded by the BMI of individuals with diabetes, as seen in the Oskar, Engmann et al. (2018) and Roubidoux, Kaur et al. (2003) studies. In addition, one of the studies observed this trend for lower MBD in both the women on metformin and the women controlling their diabetes through diet. This finding shows that no definitive statements can be made. Still, there is potential for another cause or pathway for the decreased MBD seen in diabetic women, therefore more research is required to have a greater understanding of the pathways or processes contributing to this finding and whether it is just the effect of a larger BMI on lower MBD.

1.3 Investigational Product

1.3.1 Introduction

As the tissue environment associated with MBD increases breast cancer risk, it can be deemed carcinogenic. For this reason, there needs to be interventions that can be provided to premenopausal women to reduce the lifelong exposure of the breast tissue to this carcinogenic environment (Boyd, Berman et al. 2018). The only widely accepted approach in premenopausal women is the previously described intervention of tamoxifen. As tamoxifen is a partial agonist of oestrogen, it causes significant alterations in both breast and systemic oestrogenic stimulation, the latter resulting in significant treatment-related AEs. These treatment-related AEs result in many women not complying with the tamoxifen treatment protocol; with compliance as low as 4% in women who are at increased risk of breast cancer accepting the use of tamoxifen for chemoprevention (Ropka, Keim et al. 2010).

As reported in Section 1.2.4.3 tamoxifen is the only approach for premenopausal women due to the undue effects on Ai on premenopausal women concerning ovarian stimulation. However, raising the plasma testosterone will inhibit the rise in GnRH induced from a reduction in oestrogen, thus preventing this compensation. As studies suggest that testosterone can exert a negative feedback and an inhibitory effect on GnRH secretion

(Matsumoto and Bremner 1984, Pitteloud, Dwyer et al. 2008). In the literature it has been suggested that there are two separate, more effective approaches than tamoxifen, to achieve the breast tissue E/A ratio alteration required to reduce high MBD and thus reduce breast cancer risk. Firstly, a combination of testosterone with anastrozole, a third-generation Ai, patented as HAVAH*T+Ai*™ and secondly, anastrozole with enobosarm, a selective androgen receptor modulator (SARM).

1.3.2 HAVAH*T+Ai*™

HAVAH*T+Ai*™ is the brand name for the combination of testosterone and anastrozole by HAVAH therapeutics Pty Ltd, a South Australian based pharmaceutical company. Within the breast, many enzymes convert reproductive pro-hormones, including aromatase and 5α reductase (Suzuki, Miki et al. 2006, Vachon, Sasano et al. 2011). These enzymes convert testosterone to either 17 β-oestradiol or 5α-dihydrotestosterone (DHT), the latter being ten times more potent than testosterone as an androgenic agent. High MBD tissue has been shown to contain very high levels of aromatase (Vachon, Sasano et al. 2011) resulting in enhanced intracrine production of oestrogen, even in the premenopausal breast (Dabrosin 2005).

HAVAH*T+Ai*™ utilizes the overexpression of the enzymatic systems which are especially present in high MBD tissue. By using a pharmacological dose of testosterone combined with a low dose Ai, the Ai blocks the conversion of testosterone to oestrogen, thereby increasing available testosterone in the breast tissue. This increased testosterone is hypothesised to shift the E/A ratio towards an androgenic tissue environment, which, as mentioned in section 1.2.4.1, may be favourable for reducing MBD. In addition, higher serum testosterone results in more of this androgen being delivered to the breast; ultimately, the consequence of these two actions is a high level of intramammary testosterone being made available for conversion to DHT and a reduction in intra-mammary E_2.

1.3.2.1 Clinical Rationale

Glaser and Dimitrakakis (2013) reported using a combination of subcutaneous testosterone and anastrozole implant as hormonal replacement therapy in 1,268 women. They subsequently undertook a prospective evaluation of breast cancer incidence in this cohort.

After 5,642 person-years of follow-up, the incidence of breast cancer in the cohort was 142 cases per 100,000 person-years; substantially less than the National Cancer Institute age-specific surveillance, epidemiology, and end results program (SEER) incidence rates (293/100,000), the placebo arm of Women's Health Initiative Study (300/100,000) and the never-users of hormone therapy from the Million Women Study (325/100,000) (Chlebowski, Kuller et al. 2009, Jemal, Siegel et al. 2009, Beral, Reeves et al. 2011, Glaser and Dimitrakakis 2013). This level of reduction in breast cancer risk is comparable to that seen in women who achieved a greater than 10% reduction in the MBD in the tamoxifen IBIS-I prevention trial, which is one of the seminal tamoxifen trials in the literature (Cuzick, Warwick et al. 2011).

It has also been noted that a very small amount of anastrozole could prevent gynaecomastia in men abusing large amounts of anabolic steroids for bodybuilding (Coopman and Cordonnier 2012). Therefore, it was hypothesised that using a relatively small amount of testosterone, would not result in an elevation in serum E2, due to the presence of a third-generation Ai.

1.3.2.2 Summary of Rationale for HAVAH*T+Ai*™

The rationale for therapeutic intervention with combination testosterone and Ai (HAVAH*T+Ai*™) in premenopausal women with high MBD as a marker for high risk of breast cancer is based on both non-clinical and clinical observations. With the observed ability to shift the fulcrum of the E/A ratio to affect a tissue response in the premenopausal breast that is similar to that seen in the postmenopausal breast (i.e. a low E/A ratio), which should result in a protective effect against breast carcinogenesis.

1.3.3 Anastrozole and Enobosarm

The second approach that has been hypothesised to reduce oestrogenic drive is using a selective androgen receptor modulator (SARM) as an androgen, and again an Ai to block the conversion of androstenedione to E_1 and testosterone to E_2, decreasing both serum and breast tissue oestrogen levels (Dowsett, Jones et al. 1995, Miller and Dixon 2001, Geisler, Haynes et al. 2002, Ingle, Buzdar et al. 2010).

The SARM enobosarm is currently undergoing clinical trials for breast cancer treatment and urinary incontinence. Enobosarm appears to be tissue-selective, as it maintains the anabolic actions of androgens without causing the androgenic virilising side effects, such as excess hair growth, and male type baldness, which are both commonly seen with testosterone usage (Gao and Dalton 2007). In addition, it is hypothesised that high levels of SARMs can regulate the function of the hypothalamic-pituitary axis, which includes the GnRH from the hypothalamus (Gao and Dalton 2007). This regulation will inhibit the hypothalamic-pituitary overstimulation; therefore, allowing the Ai to function fully within the breast tissue, without having a meaningful impact on other peripheral tissues which are dependent on oestrogens action.

1.4 Mammographic Breast Density as a Baseline Risk Marker and Surrogate Endpoint for Breast Cancer

As there is a strong association between MBD and the risk of breast cancer, it allows MBD to be used as a biomarker to evaluate the efficacy of interventions that are aimed at reducing breast cancer risk. In the design process of clinical trials, one of the most important considerations needs to be the choice of outcome measure or measures. These outcome measures can be clinically meaningful endpoints that are direct measures of how a patient feels, functions and survival rates. Alternatively, indirect measures can be used, an example of these are biomarkers, which can include physical signs of disease, laboratory measures and radiological tests, which may be considered as replacement (or surrogate) endpoints for clinically meaningful endpoints (Fleming and Powers 2012). The changes induced by a therapy on a biomarker or surrogate endpoint are expected to correlate and reflect the changes in clinically meaningful endpoints (Temple 1995). These endpoints or biomarkers should have the properties of being well defined, reliable, easily measurable and interpretable, and be sensitive to the effects of an intervention. The sensitivity is usually a leading factor in the selection of an outcome measure; thus, enabling a reduction in the size and duration of clinical trials, to aid the achievement of significant results (if significant results are to be seen) (Fleming and Powers 2012).

Mammographic breast density is one of the most commonly accepted biomarkers for breast cancer risk in the literature (Heine and Malhotra 2002, McCormack and dos Santos Silva 2006,

Boyd, Guo et al. 2007, Vachon, Van Gils et al. 2007). McCormack and dos Santos Silva (2006) found that in symptomatic women, there was little evidence of interactions between other risk factors for cancer and MBD. The data they combined suggests that MBD in both women of premenopausal and postmenopausal ages is a marker of subsequent breast cancer risk, the evidence of whether the strength of this association differs between the ages is not clear. In addition, the potential for MBD to guide breast cancer interventions, as opposed to tissue and circulating biomarkers (Sivasubramanian and Crew 2013), is particularly appealing since MBD significantly correlates with both breast cancer risk and outcomes. Specifically, MBD changes in response to some endocrine manipulations, it is non-invasive, and may easily be incorporated in the routine care already employed in screening and follow up tools for breast cancer; which minimises cost and effort for the patient (Shawky, Martin et al. 2016).

The promise of the research utility of MBD and the lack of timely alternatives have encouraged researchers to consider MBD as a surrogate endpoint for risk of breast cancer events (Guerrieri-Gonzaga, Robertson et al. 2006). This potential of MBD as a surrogate endpoint has lead to MBD being used in clinical trials as a secondary endpoint (Decensi, Gandini et al. 2007) and as a primary endpoint (Birmingham 2000); this also including studies that are researching Ais in the preventative setting.

1.4.1 Issues with Using Mammographic Breast Density and its Changes as a Breast Cancer Biomarker

Even though there is a strong association between MBD and breast cancer, there are several limitations that may affect the clinical and research utility of this outcome measure. Below are some of the reasons why this is the case.

1.4.1.1 Imaging Technique and Interpretation

If MBD has been calculated using a subjective non-automated method (e.g. Wolfe's system, BI-RAD score or breast density estimation using a quantitative area-based analysis), the results can have a high intra-and inter-reader variability (Ooms, Zonderland et al. 2007, Succurro, Arturi et al. 2010). These methods may also lack the precision to see a significant change in MBD, which can lead to type 2 errors (otherwise known as a false negative) and insignificant results in clinical trials. Furthermore, type 2 errors can lead to beneficial and

viable treatments being unavailable to patients, a loss of development costs and the potential loss of profits. The more recent, fully automated volumetric estimation methods (such as VolparaDensity™), which show the thickness of dense tissue at each pixel on the mammogram (Ng and Lau 2015), are able to show a more objective measurement and may reveal more sensitive and significant changes. However, these automated methods also have some limitations. A correlation has been established between the increasing image quality of mammograms, whether this being due to advancing imaging technologies or with different radiographers taking the image, having an effect on the generated results (Kerlikowske 2007, Vachon, Pankratz et al. 2007, Lokate, Stellato et al. 2013, Work, Reimers et al. 2014). This effect on the results can be problematic in longitudinal studies or when clinically monitoring MBD over a period of time, as changes seen in the image and reported on may not reflect the changes occurring in the breast tissue.

1.4.1.2 Breast Pain with Mammography

Breast pain is a prevalent condition amongst women (Masood, Ader et al. 1998) and several studies have reported that a women's fear of pain during mammography is a significant barrier to attending mammography for the first time (Kee, Telford et al. 1992, Straughan and Seow 1995). In particular, the fear of the pain that will arise from the compression of the breast (Straughan and Seow 1995). In addition, two small studies found that pain during first-round mammography is also a major barrier for re-attendance (Marshall 1994, Elwood, McNoe et al. 1998). In these two studies, pain was the highest reason for not re-attending breast screening. It could be assumed that this phenomenon may be replicated in research, and increase attrition rates in clinical trials, which results in incomplete data acquisition.

1.4.1.3 Ionising Radiation

A mammogram requires a woman to be exposed to ionising radiation in order to generate the image. Ionising radiation itself is a risk factor for breast cancer; a doubling of the risk of breast cancer has been observed among teenage girls who were exposed to radiation during the Second World War compared to women who weren't exposed (McPherson, Steel et al. 2000). Ionising radiation also increases breast cancer risk later in life, and this is particularly problematic when exposure has occurred during rapid breast formation (McPherson, Steel et al. 2000). Due to powerful new imaging techniques, the per capita dose of ionising radiation

used for medical imaging procedures has increased six-fold between the 1980s and the present (Mettler Jr, Bhargavan et al. 2009). In a cohort of 100,000 women, mammographic screening that was conducted annually from ages 40 to 55 years and biennially until age 74 years at a dose of 3.7 milligrays per examination would ultimately induce 86 breast cancers (Yaffe and Mainprize 2011). If MBD is to be utilized in premenopausal women as a biomarker of risk and therapeutic efficacy, frequent mammograms would be required. This increased frequency would also increase the ionising radiation exposure; this should ideally be avoided to reduce the risk of radiation-induced breast cancers.

1.4.1.4 Outcome Measure Sensitivity

The sensitivity to detect the efficacy of an intervention may be inadequate with all MBD measures. Cuzick, Warwick et al. (2004) reported that women taking tamoxifen, as a chemopreventative agent due to being classified as high breast cancer risk, had an absolute mean decrease in MBD of 7.9% (relative decrease of 18.9%) at 18 months and 13.7% (relative decrease of 32.7%) at 52 months. Chow, Venzon et al. (2000) also reported that women on tamoxifen therapy have an average yearly relative reduction in MBD of 4.3%. Both of these studies demonstrate that MBD is either slow to respond to treatment, and elicit measurable changes or the intervals typically used for mammography are longer in duration and may not show changes in a timely manner. For this reason, to adequately study MBD modifications, long, expensive trials are needed to determine the efficacy of chemopreventative agents or to determine if women are responders or non-responders to a therapeutic intervention.

1.4.1.5 Conclusion

These limitations with mammography and the measures of MBD demonstrate that it would be beneficial to validate a biomarker for MBD to determine the response within the breast tissue to therapeutic interventions. As there is a substantial number of women developing either benign or malignant breast disease, there needs to be a focus on research technologies and biomarkers that assist in determining the effectiveness in preventative and targeted interventions so they can be thoroughly evaluated and brought to market. This biomarker needs to be sensitive to detect change, which ideally would be in a timely manner, therefore, can benefit research and clinical uses. This timely outcome measure would allow the healthcare provider to modify the treatment based on whether the patients are responders

or non-responders; which can greatly influence the patient's management and their health-related outcomes. Reliable and valid biomarkers are also beneficial regarding health economics; the ability to detect the efficacy of a drug allows resources to be applied more efficiently and costs to be saved if an intervention is deemed ineffective (Manton, Chaturvedi et al. 2006). Traditionally, especially in oncology, but across other medical fields, a patient's response to their treatments have been assessed via a variety of techniques including clinical palpation, x-ray mammography, ultrasound and magnetic resonance imaging (Pickles, Gibbs et al. 2006). Unfortunately, the assessment of treatment response via these approaches can be considered to be a late event, since functional changes occur within the tumour before changes in the size of the tumours or global tissue changes (Chenevert, Meyer et al. 2002, Hayes, Padhani et al. 2002, Padhani 2002).

1.5 Breast Tissue Elasticity

Within this research program, it was hypothesised that breast tissue elasticity may be an innovative biomarker for MBD and an appropriate method to measure the changes in the breast tissue in response to preventative or targeted therapeutic interventions. The following sections will introduce the biomechanical properties of breast tissue elasticity to justify the reasoning behind the overarching focus of this doctorate thesis.

1.5.1 Tissue Elasticity Basics

Elasticity is the measure of the stiffness of a material. When a material is deformed, if it returns to its original shape, it is deemed to be elastic. The opposite of this is plastic, which is when the material is deformed; it maintains the deformed shape. Soft tissues and their biomechanical properties are dependent on the inherent molecular building blocks (fat, collagen, and fluid-filled sacks) and the microscopic structural organisation of these building blocks (Fung 1981). The notion of tissue elasticity has been present in health care for an extended period of time and is used in a variety of different settings and professions; the common practice of tissue palpation being based on the subjective assessment of tissue elasticity (Ophir, Alam et al. 2002). During palpation, the fingers push the tissue downwards (displace the tissue) and the pressure receptors on one's finger can detect the differences in the local stiffness (elasticity) of the tissues (Hall 2003). The sensation felt when palpating a

hard lesion is due to higher elasticity values locally, which is then lower for areas overlying softer surrounding tissues (Hall 2003). Although this is useful in some elements of clinical practice, this technique is limited as it is subjective, and the examiner is unable to quantify the elasticity values and may not be able to accurately detect elasticity changes.

The Young's Modulus is an equation used that can describe the change in length of material concerning stretching or compressive forces (Garra 2007); this is the classic parameter to describe the elasticity of tissues. This parameter is mathematically defined as:

$$E = \frac{F}{A}\frac{L_0}{\Delta L} \; or: \; \frac{F}{A} = E\,\frac{\Delta L}{L_0}$$

Within this equation, *E* is the Young's Modulus, *F* is the force, *A* is the area over which the force is applied, ΔL is the change in the length in response to the force and L_0 is the original length of the object or material (Garra 2007). This means that when an external uniform compression (the stress) is applied to a solid tissue, a deformation (or strain) is produced inside the tissue. The Young's Modulus is stress divided by strain and can quantify the tissue stiffness; hard tissues have a higher Young's Modulus and softer tissues a lesser. The unit used to quantify the stress and Young's Modulus is the pascal (Pa, where one Pa= 1 Newton per square meter (N/m^2) or more commonly the kilopascal (kPa).

1.5.2 Elasticity Imaging

The predominant method of breast elasticity quantification is a medical imaging technology called elastography. Elastography is an encouraging form of medical imaging in health care, as inevitably the biomechanical properties of soft tissues are linked to the tissues overall health (Sarvazyan, Rudenko et al. 1998) with pathological changes including inflammation, wound healing, and cancer being correlated with changes in the tissue elasticity (Ophir, Alam et al. 2002). As mentioned, the pathological changes can be detected with clinical palpation and the sensation of stiffer tissue underlying the clinician's fingers with malignant masses compared to 'normal' tissue. Elastography is currently being used for several clinical and health research applications for breast tissue and breast conditions. The predominate use of elastography for breast tissue currently being presented in the literature is the differentiation of benign and malignant breast lesions (Athanasiou, Tardivon et al. 2010, Chang, Moon et al.

2011, Berg, Cosgrove et al. 2012, Au, Ghai et al. 2014) with the aim of reducing the number of required diagnostic biopsies. It could be argued that this lacks clinical utility as patients may predominately want histological findings to confirm or negate a malignant diagnosis. In addition, research has also shown that 6.4% to 36.6% of benign or malignant breast masses have elasticity values which do not conform to their histopathologic diagnosis. Therefore, these elasticity values may lead to false-negative results which can reduce the sensitivity of elastography as a diagnostic tool (Chang, Moon et al. 2011, Gweon, Youk et al. 2013, Yoon, Jung et al. 2013). There is currently limited research into other clinical application of breast elastography (these are listed in Section 1.5.2.3). Still, there is the potential that tissue elasticity may be able to be used as a biomarker for the efficacy, and as a prognostic guide for therapeutic interventions within the field of breast health. Specifically, the focus of this thesis is to discover if breast elasticity has the potential to be used as a biomarker for MBD.

1.5.2.1 Elasticity Imaging: Shear Wave Elastography

As introduced in Section 1.5, elastography is the term used to refer to the imaging techniques that aim to assess tissue elasticity. Elastography depicts the stiffness of the tissues, which allows for an objective, quantitative estimation of the tissue elasticity, independent of its morphological features. A variety of techniques and approaches have been utilised for advancing elastography imaging, regarding both applying the force and for measuring and displaying the tissue response (Nightingale, McAleavey et al. 2003, Bamber, Cosgrove et al. 2013). One measure of elastography is shear wave elastography (SWE), which can be utilised to produce two or three-dimensional quantitative ultrasound images (Bamber, Cosgrove et al. 2013) and can provide a colour coded real-time, objective measure of breast tissue elasticity in the unit of the kPa.

Shear wave elastography is conducted by having ultrasound beams generate acoustic radiation force impulses, which provide the mechanical excitation through pushing beams that deform the underlying tissue of interest. Several of these pushing beams are transmitted at different depths, which results in the propagation of transient shear waves. The speed of these shear waves is then measured using a scanner with a very fast frame rate, allowing the shear waves to be followed in real-time. This is repeated for different lines; allowing a map of a region of interest (ROI) to be created from analysing the differences in arrival times and calculating the shear wave speeds. A colour-coded image is then displayed on the SWE

monitor, and the quantitative data is presented as a measure of shear wave speed in meters per second (m/s^{-1}) or converted to the Young's Modulus and displayed as kPa. Throughout the measurement, the shear wave imaging is adjunct to the B-mode image and guidance is possible as the same transducer that generates the shear waves also captures their propagation (Bercoff, Tanter et al. 2004, Sebag, Vaillant-Lombard et al. 2010, Shiina, Nightingale et al. 2015).

1.5.2.2 Elasticity and Shear Wave Elastography in Current Clinical Practice

It has generally been agreed upon in health care, that no other physical parameters of tissue change to as great an extent, with physiological and pathological influence, as does elasticity (Manduca, Oliphant et al. 2001). This statement suggesting that SWE has favourable properties as an outcome measure in clinical practice. Typical values of breast elasticity have been reported (Skovoroda, Klishko et al. 1995, Sarvazyan 2001, Duck 2013) and are summarised in Table 1-4. It could be hypothesised that breast pathologies fall within a spectrum of tissue elasticity values, with lower elasticity representing normal tissue and tissues in a pathological state become stiffer, trending with higher elasticity values.

Table 1-4: Summary of typical breast elasticity values

Area	Type of Soft Tissue	Elasticity in kPa
Breast	Normal Fat	18-24
	Normal Glandular	26-66
	Fibrous Tissue	96-244
	Carcinoma	22-560

Currently, as mentioned, the primary clinical and research focus of elastography in breast tissue has been differentiating lesions as being either benign or malignant. The current research showing that there is a statistically significant difference in elasticity values between these two lesions types, in which malignant lesions have a higher elasticity value (Athanasiou, Tardivon et al. 2010, Chang, Moon et al. 2011, Berg, Cosgrove et al. 2012, Au, Ghai et al. 2014). However, Chang, Moon et al. 2011 determined, using a sample of 162 consecutive women with 186 needle biopsied or surgically excised lesions, that SWE had a sensitivity of 88.8% and specificity of 84.9% in differentiating benign or malignant breast lesions. This diagnostic accuracy is too inaccurate for SWE to be used independently as a diagnostic tool. Additionally, some varieties of malignant tumours have differing biomechanical properties,

and as a consequence, elasticity values are more representative of a benign growth (Falou, Sadeghi-Naini et al. 2013). Furthermore, patients are becoming more adept at taking control of their healthcare and in cases where clinical management decisions and prognosis hinge on the pathology of the tissue in question, it is vital to have confidence in the diagnosis. Patient and families may not be satisfied with a mass being diagnosed as benign without any histological studies on the tissue in question. These factors have the potential to hinder its use as a routine clinical application within this area. There are, however, several other applications that SWE can be used in for the diagnosis and management of breast health.

1.5.2.3 Alternative Applications of Shear Wave Elastography

1.5.2.3.1 Shear Wave Elastography and Neoadjuvant Chemotherapy

One encouraging area for the use of SWE is the prediction and monitoring of the tissue response in women undergoing neoadjuvant chemotherapy (NAC) for malignant breast lesions. Overall, the response to NAC is variable between patients, with approximately 77% of patients having a positive response (termed being a responder) to the therapy (Lee, Seo et al. 2013). Due to this variability, early evaluation of the response is crucial to improving patient's health-related outcomes and decreasing the financial cost of the patient management. During treatment, the disease may progress which can result in a delay regarding the optimal time for surgical intervention (Jing, Cheng et al. 2016) and early identification of unresponsive tumours can lead to prompt changes in the patient management. This, in turn, can lessen the unwarranted side effects of unnecessary or non-beneficial drugs and improve the prognosis of the patient (Jing, Cheng et al. 2016). Furthermore, the formulation of a management plan with a validated biomarker to predict/determine an individual's response to NAC is becoming a priority in breast cancer research (Cho, Im et al. 2016). Recent studies have highlighted the importance of early detection of patients not responding to NAC, as it has been demonstrated that delivering radiation and surgical intervention for chemotherapy-resistant tumours can result in a survival rate of 46% at five years (Huang, McNeese et al. 2002).

Currently, clinical examinations in combination with traditional imaging techniques such as computer tomography (CT), MRI and mammography may be used to predict and evaluate the response of the tumour to NAC (Hylton, Blume et al. 2012, Falou, Sadeghi-Naini et al. 2013, Li, Arlinghaus et al. 2014, Jing, Cheng et al. 2016). These methods are often unable to provide an

objective evaluation of the response during the early phase of treatment, as previously reported in Section 1.4, functional changes relating to microscopically evident tumour death may occur before macroscopic or global tissue changes (Falou, Sadeghi-Naini et al. 2013). In addition, methods of cell death induction, such as chemotherapy, can substantially alter the biomechanical properties of the malignant tissues during a course of treatments (Wang, Guo et al. 2018). This is mainly because tumour formation and the degeneration in response to treatment exhibits significant interactions, e.g. fibrosis and inflammation with stromal cells (Mueller and Fusenig 2004, Schedin, O'Brien et al. 2007); having the ability to change the biomechanical properties of these cells. Furthermore, results from animal laboratory studies have indicated that the stiffness of a tumour is related to the tumour progression and chemotherapeutic resistance (Butcher, Alliston et al. 2009). Therefore, with SWE's ability to objectively provide a measure of the tumour stiffness, it allows the potential for predicting and evaluating the response to NAC in individuals with breast cancer (Jing, Cheng et al. 2016). Shear wave elastography is also less expensive than other imaging techniques, and it does not require the use of contrast agents, which is well suited for the multiple scans that are necessary to monitor this form of treatment. On this basis, SWE has physiological merit to assess the effectiveness of NAC between responding and non-responding malignant tissues, with an early time frame of a few weeks, following the start of the therapy.

To date, a few studies have investigated this utility for alternative elastography imaging techniques and in more recent times SWE. Falou, Sadeghi-Naini et al. (2013) using strain elastography (an alternative form of elastography), evaluated the responses to NAC in 15 women with locally advanced breast cancer. Within this study, the authors found that the strain ratio of the tissue stiffness was the best predictor of the response to NAC treatment. The findings also revealed, that after the baseline assessment and after the second cycle of NAC, the tumours of those who responded to treatment were significantly softer than those of non-responders. However, this study also demonstrated that with a rare form of cancer (mucinous cancers), the SWE measurements lack sensitivity, this being due to the biomechanical properties of the growth; in particular the abundant extracellular mucin (Falou, Sadeghi-Naini et al. 2013).

Fang and Yang (2019) also using strain elastography conducted a study to explore the value of real-time tissue elastography in predicting the efficacy of NAC. The study had two groups of

women; one group had a significant response to NAC (the responders) and one who did not have a significant response (the non-responders). The authors reported that grayscale ultrasound was not able to accurately evaluate the efficacy of NAC. It was also found that the elasticity of the tumour decreased in both groups up to 2 weeks. At 2 weeks the non-responders tumour elasticity plateaued as did the size of the tumour, the responder's elasticity continued to decline. The results also showed that the elasticity changes closely related to the size and state of the lesion, which demonstrates that the elasticity values post the two-week mark may be able to assist in determining if a woman is going to be a responder or a non-responder to the treatment (Fang and Yang 2019).

Similarly, Jing, Cheng et al. (2016) conducted a study which examined the use of SWE for the early prediction of the response to NAC in women with breast cancer. The authors found that in relation to the baseline values of the tumour stiffness following two cycles of NAC, the stiffness was decreased and the change was significantly different in the responders (mean elasticity after the second cycle: 50.18 kPa ± 25.01 kPa) but not in the non-responders (mean elasticity after the second cycle: 80.37 kPa ± 27.18 kPa). In addition, there was a significantly greater change in tumour stiffness after the second cycle of NAC in the responders (-42.19% ± 19.99%) than in the non-responders (-23.59% ± 8.22%). Whether it was assessed at the baseline measurement or after the second cycle of NAC, there was a significantly lower mean tumour stiffness in the responders (baseline kPa 82.76 ± 47.43) compared to the non-responders (baseline kPa 99.77 ± 45.45). The results showed that the area under the receiver-operated curve for tumour stiffness was 0.80 ($P<0.001$) which indicated that within this study, tumour stiffness represented a useful tool for predicting and determining the neoadjuvant response of the breast cancer to the therapy. Ma, Zhang et al. (2017) had similar findings within their research with invasive breast cancers, reporting that tumours with lower stiffness values at baseline displayed better NAC responses and more frequently favourable pathological responses compared to stiffer tissues, and after the second cycle of NAC SWE could provide early prediction of the pathological resistance to NAC with the stiffness of the tumours.

Supporting this finding, Evans, Armstrong et al. (2013) found that there was a statistically significant relationship between the baseline (pre-treatment) tissue elasticity with the response of the invasive breast cancer to NAC and subsequently the levels of residual cancers.

The findings were demonstrating that the stiffer tissues measured at baseline responded to NAC to a lesser extent than softer tissues. These results follow previous results by Hayashi, Yamamoto et al. (2012), who used strain elastography in 55 patients who received NAC for breast cancer and found that there was a close association between the tumour stiffness and the response to NAC in breast cancers. The findings demonstrating that relatively soft tumours were highly responsive to NAC and more frequently displayed complete resolution compared with stiffer tumours (complete resolution rate 50 vs 14%, respectively).

Finally, Lee, Chang et al. (2015) evaluated the accuracy of SWE in the detection of residual breast cancer after NAC and discovered that women with residual cancers showed significantly higher maximum elasticity values (mean kPa 116.0 ± 74.1) than women who achieved complete resolution (mean kPa 26.4 ± 21.0). Additionally, Lee, Chang et al. (2015) found that the diagnostic performance was highest when using MRI compared to B-mode ultrasound and SWE; however, the difference between MRI and SWE was not statistically significant. In addition, SWE significantly improved the diagnostic performance of B-mode ultrasound regarding the detection of residual breast cancers.

These studies demonstrate that responders to NAC may initially have softer tumours and show more significant changes in tissue elasticity values through the treatment process than the non-responders. Additionally, individuals with softer tumours are proving to have a higher likelihood of having complete resolution with treatment than those with stiffer tissues. The early stages of research are showing that SWE has the potential to be a viable and robust addition and a promising biomarker for the research and clinical practice of determining the prediction and response of breast tumours to NAC.

1.5.2.3.2 Shear Wave Elastography in Breast Inflammation

Mastalgia (or breast pain), is a common clinical occurrence in most women during their reproductive life and occasionally after menopause. In approximately 15-20% of women, breast pain is of a severity to impact lifestyle and requires intervention (Scurr, Hedger et al. 2014). One of the causes of breast pain appears to be related to hormonally induced inflammation (Fentiman, Caleffi et al. 1988); to overcome this inflammation, the underlying hormonal imbalance needs to be managed. In similar clinical circumstances as NAC, SWE may

be a viable biomarker to monitor the inflammatory changes within the breast tissue. It is hypothesised that inflammation leads to a greater elasticity. Hence, as the inflammation decreases theoretically, the pressure within the tissue should decrease, resulting in lower elasticity values as recorded by SWE.

To date, SWE has been used in two studies on patients with mastitis, which is an inflammatory condition of the breast that may either be infectious or non-infectious in origin (Sousaris and Barr 2016). Typically, women suffering from mastitis have oedema and breast pain caused by infectious or chemically induced inflammation. Sousaris and Barr (2016) reviewed six cases of biopsy-proven mastitis; the results indicated that the mastitis could either have a central soft area (the centre of an abscess) and a stiff outer region (caused by oedema and inflammation) or just the stiff outer region. The stiff outer region of the tissue has a stiffness range of 35-120 kPa with a mean value of 72.0 kPa, which is higher than the elasticity range of disease-free breast tissue, as stated in Section 1.5.2.2. Furthermore, Ko, Jung et al. (2014) within a study on non-malignant breast lesions included one case of chronic mastitis, again using SWE, found this tissue also had an elevated mean stiffness of 59.3 kPa.

These results are based on a small sample size; therefore, correlations and the interpretation of the data needs to be done with caution. However, these preliminary findings demonstrate that elasticity values may trend higher in breast tissue where inflammation is present. With future research, looking at a greater sample size, it is possible that SWE could have promise to be used as a biomarker for mastitis or other inflammatory conditions, to determine the therapeutic efficacy of interventions to reduce general inflammation and hormonally driven breast inflammation in the clinical and research setting.

1.5.2.3.3 Shear Wave Elastography in Breast Augmentation, Mastectomy and Reconstruction Surgery

Shear wave elastography may also have clinical utility for the evaluation of contractures, pain and inflammation in relation to breast augmentation surgery. Following breast augmentation surgery, it is normal for a capsule to form due to the natural inflammatory response to a foreign object entering the body, such as a breast implant. If this happens beyond the typical state peri-implant fibrosis and capsular contraction may occur. This can lead to tissue

distortion, hardness, and pain (Basu, Leong et al. 2010). Currently, the Baker scale, which is a subjective scale based on a clinical evaluation of appearance, texture and tenderness, is used to classify the severity of the capsular contraction (Basu, Leong et al. 2010). As the biomechanical properties of the breast tissue changes with capsular contraction; tissue elasticity may be a viable method of objective evaluation for this condition. This evaluation could be beneficial as up to 30% of patients with the two highest grades of contractures potentially require surgery (Basu, Leong et al. 2010), early assessment and the ability to objectively detect excessive capsular formation would be of great benefit to improve clinical practice and patient outcomes.

Three papers to date have been published in the field of SWE in the evaluation of breast capsular contractures. A two-person case study by Rzymski, Kubasik et al. (2011) reported that one of the cases, who had a Baker III contracture in their left breast and a Baker I contracture in their right breast, showed that post-implant replacement and capsulectomy, there was a decrease in tissue elasticity in the breast area (left breast 27.3 reduced to 20.5 kPa and right breast 15.3 reduced to 14.9 kPa). The second case, who had a Baker I contracture in their left breast and Baker III/IV in their right showed an increase in elasticity values (left breast 12.5 increased to 23.5 kPa and right breast 17.6 increased to 22.4 kPa). This patient, however, in contrast to patient one, had an additional 85ml of fluid inserted into their left breast and 95ml of fluid inserted into the right; which may have influenced the elasticity values. These findings demonstrating that within these two subjects the Baker III contractures had higher elasticity values than the Baker I contractures however the findings indicate that post capsulectomy and implant replacement the values can be influenced by the fluid volume of the implant if not kept consistent. Rzymski, Kubasik et al. (2011) also reported that almost all tissues (fatty, glandular, fascia, and muscles) have elasticity values 2-4 times higher on day ten after primary breast augmentation and tend to decrease on day 20. Further research could be conducted to assess if the changes in elasticity with the healing process would allow potential tracking of this elasticity to detect complications during recovery from this type of surgery.

Sowa, Yokota et al. (2017) also used SWE for the measurement of capsular contracture after breast implant reconstruction, with 20 patients (27 implants) the authors reported that elasticity values were strongly correlated to the Baker Score with a correlation coefficient of

0.81, and the reproducibility showed an intra-class correlation coefficient of 0.88, which is a high-reliability score. This correlation coefficient demonstrates that within this study, SWE was a highly reproducible method of detecting the degree of capsule contracture, deeming it a potentially useful clinical tool post breast reconstruction, if being done by the same clinician.

Furthermore, Rzymski, Kubasik et al. (2011) observed that when using SWE there were statistically significant changes in all breast tissues with the highest values being recorded on day seven post-surgery; this elasticity was then found to decrease on day 14. The authors reported that between days four and ten there were significant correlations between the visual analogue scale (VAS) for pain and the capsular elasticity in the lower quadrants; this correlation was not found within the glandular tissues in the same quadrant. However, in the upper quadrants, where the glandular tissue concentration is higher, there was a significant correlation with the VAS for pain during days 6-10. Fatty tissue stiffness did not correlate with breast pain in any quadrant of the breast. Capsular contracture has been associated with a higher risk of reported pain; the authors have hypothesised that the cause of the pain was likely to be inflammatory. An objective tool, whether elasticity imaging or histological findings of inflammation, could demonstrate the bridging link between the two (Sperlingl, Høimyrl et al. 2011).

In addition to capsular contraction, SWE has been used in a small study by Sowa, Numajiri et al. (2015) to investigate fatty stiffness post breast reconstruction following a mastectomy, as fatty induration is associated with necrosis and is a common complication in breast reconstruction with autologous flaps after mastectomy (Kroll 2000, Casey, Rebecca et al. 2013). Currently, within the clinical setting, palpation is used, which is a subjective measure and, as previously mentioned, is unable to be quantified. The authors found that in one case study, in the superior medial area of the breast, fatty tissue showed increased stiffness (mean 22.3 kPa) when compared to the lateral area (mean 6.6kPa). Furthermore, another case who complained of a breast mass with pain and stiffness had a significantly higher SWE reading (mean 107.4 kPa) in the superior medial area compared to the lateral area (13.9kPa) finding that the breast mass was associated with fat necrosis.

These preliminary results based on small sample size suggest that with further research SWE may be able to facilitate post-surgical care and management and may be accepted into clinical practice post breast augmentation or reconstruction. Although still in the early phase of research, there is potential that SWE will be able to be used to offer new possibilities of postoperative follow up; determining if elasticity values fall in the expected timeline or remain stagnant or continue to increase can provide clinicians with valuable information leading to the prediction that unwanted complications may be occurring and provide the opportunity for early intervention.

1.5.2.3.4 Breast Elasticity as an Alternative Biomarker of Mammographic Breast Density

Breast elasticity, as measured by SWE, may have the potential to be used as a biomarker to determine the effectiveness of therapeutic and preventative interventions which are aimed at reducing breast cancer risk. Breast elasticity also has the potential to be a correlated measure of baseline MBD, which may be beneficial to guide clinical reasoning and decision making for referring a patient to begin mammography screening. It is presently known that breasts that have a high MBD are associated with extensive collagen, a greater number of cells, increased extracellular matrix (ECM), including the increased expression of the proteoglycan lumican and decorin (Alowami, Troup et al. 2003, Li, Sun et al. 2005, DeFilippis, Chang et al. 2012). These proteoglycans can bind growth factors, which contribute to the mechanical integrity of tissues; influencing the elasticity and the behaviour of the breast tissue (Butcher, Alliston et al. 2009). It has also previously been reported that high MBD tissue shares similar characteristics to malignant breast tissue, specifically fibrodense areas having high ECM content and low adipocytes (DeFilippis, Chang et al. 2012). This abnormal ECM deposition can lead to tissue stiffening, causing greater tissue elasticity as seen in breast cancer (Bonnans, Chou et al. 2014).

Additionally, the tissue associated with increased MBD has some similar properties to individuals with hepatic fibrosis; a resultant condition of the healing response to repeated liver injury (Friedman 2003). The process of hepatic fibrosis is associated with the liver's inflammatory response and the deposition of ECM. If the hepatic injury persists, the liver's ability to regenerate begins to fail, and the usual generation of hepatocytes are substituted

with abundant ECM, including fibrillary collagen (Bataller and Brenner 2005). The accumulation of the abundant ECM in hepatic fibrosis can result from two pathways; an increased synthesis and from decreased degradation of ECM (Arthur 2000); with the decreased activity of ECM-removing matrix metalloproteinases (MMPs) mainly being due to an overexpression of their specific inhibitors (tissue inhibitors of metalloproteinase (TIMP) (Bataller and Brenner 2005). Both of these pathways have also been seen in mammographically dense tissue with Guo, Martin et al. (2001) demonstrating that within high MBD tissue, there is increased TIMP-3 expression and a positive association with metalloproteinase-3 (MMP-3).

Additionally, isoprostanes and malondialdehyde (MDA), both of which are *in vivo* biomarkers for oxidative stress, have demonstrated to be mediators for the increased cell proliferation and collagen production in hepatic fibrosis (Comporti, Arezzini et al. 2005). Oxidative stress occurs when there is an imbalance between reactive oxygen species (a collective term for oxygen free radicals or non-radical oxidising agents that can be converted easily into radicals (Halliwell and Gutridge 1989)) production and the antioxidant defences, which can lead to damaged DNA, protein and lipid molecules. Mutagenesis can occur due to DNA damage, and this can increase the risk of cancer (Valko, Izakovic et al. 2004). Inflammation has also been linked to reactive oxygen species, and maybe another reason oxidative stress relates to cancer (Pathak, Sharma et al. 2005). In three independent studies (Boyd and McGuire 1990, Boyd, Connelly et al. 1995, Hong, Tang et al. 2004), a positive association was found between MBD and 24-hour urinary MDA excretion. Additionally, in both pre- and postmenopausal women, representing a range of MBDs (Boyd, Connelly et al. 1995, Hong, Tang et al. 2004), adjusting for differences in age, BMI and waist circumference, urinary MDA excretion was 23% to 30% higher in the highest quartile of MBD when compared with the lowest MBD quartile.

Liver fibrosis is a response to tissue injury and the inflammation associated with the injury (which as mentioned previously increases oxidative stress). The processes involved are the proliferation and activation of fibroblasts, with an accumulation of ECM the resultant effect (Hinz 2007). As there are strong similarities between hepatic fibrosis and MBD, it could be hypothesised that breast elasticity may be increased in women with high MBD. As the FibroScan® (transient elastography) began as a biomarker and then a diagnostic tool for liver

fibrosis, there is potential that breast elasticity may be able to do the same for MBD. If this research does find an association between the two, breast elasticity as measured by SWE may be able to be used as a biomarker and detect changes in the breast tissue, which may also be at an earlier time, than mammographic imagery. Thus, potentially providing useful information again concerning treatment responses and monitoring breast cancer risk.

1.6 Introduction Summary

Through this research program, the performance characteristics of using SWE to measure whole breast elasticity, in relation to the clinical utility and psychometric properties for the use of breast elasticity as a biomarker for MBD, will be examined. This research program will also establish and describe a user-friendly, reliable method for using the SWE ultrasound for this indication. The benefits of conducting and reporting this evidence are firstly; it will begin to develop the body of evidence regarding the validation of breast elasticity as a biomarker in health research, in particularly pharmacology research. In addition, by providing the methodology for using SWE for this indication, researchers who wish to conduct future research in this field will not need to establish a reliable method for collecting the SWE data, which can save time and resources. Furthermore, by using a consistent methodology for SWE, evidence can be easily compared and synthesised to further validate breast elasticity as a biomarker for therapeutic interventions for the breast.

Chapter 2 Research Aims, Objectives and Structure

2.1 Aims

The primary aim of this research program is to determine if whole breast elasticity, as measured by SWE, can be used as a biomarker for MBD.

A secondary aim of this research is to determine a reliable and valid protocol when using SWE to measure whole breast elasticity in order to increase research and clinical implementation.

2.2 Objectives

1. Determine the efficacy of hormonal interventions to reduce mammographic breast density

The initial objective of this research was to determine the efficacy of HAVAH*T+Ai*™ and the combination of enobosarm and anastrozole in their ability to reduce MBD. This was conducted to provide baseline data regarding the effect of these interventions on the breast tissue, to show that there are physiological changes occurring in the primary endpoint of interest.

2. Determine the effect of these two, hormonal combination on breast elasticity and whether these correlate with changes to mammographic breast density

The second objective of this research was to determine if the two hormonal combinations (HAVAH*T+Ai*™ and the combination of enobosarm and anastrozole) can influenced breast elasticity, as measured by SWE.

3. Determine the normative values and behaviour of breast elasticity in healthy women not on any form of hormonal intervention

The third objective was to analyse the average breast elasticity of healthy women who were not on hormonal interventions that may influence the elasticity values. This was done with repeat measures to analyse the behaviour and fluctuations of the breast elasticity and whether this is influenced by hormonal changes that occur with the menstrual cycle.

4. Determine a valid and reliable protocol for the shear wave elastography machine to measure whole breast elasticity

The fourth objective was to determine if there is a reliable and precise method to determine the whole breast elasticity as measured by SWE as to date there is no consistent protocol to be used in clinical and research purposes.

2.3 Research Plan with Associated Objectives

The research plan and associated objectives are presented in Figure 2-1 below.

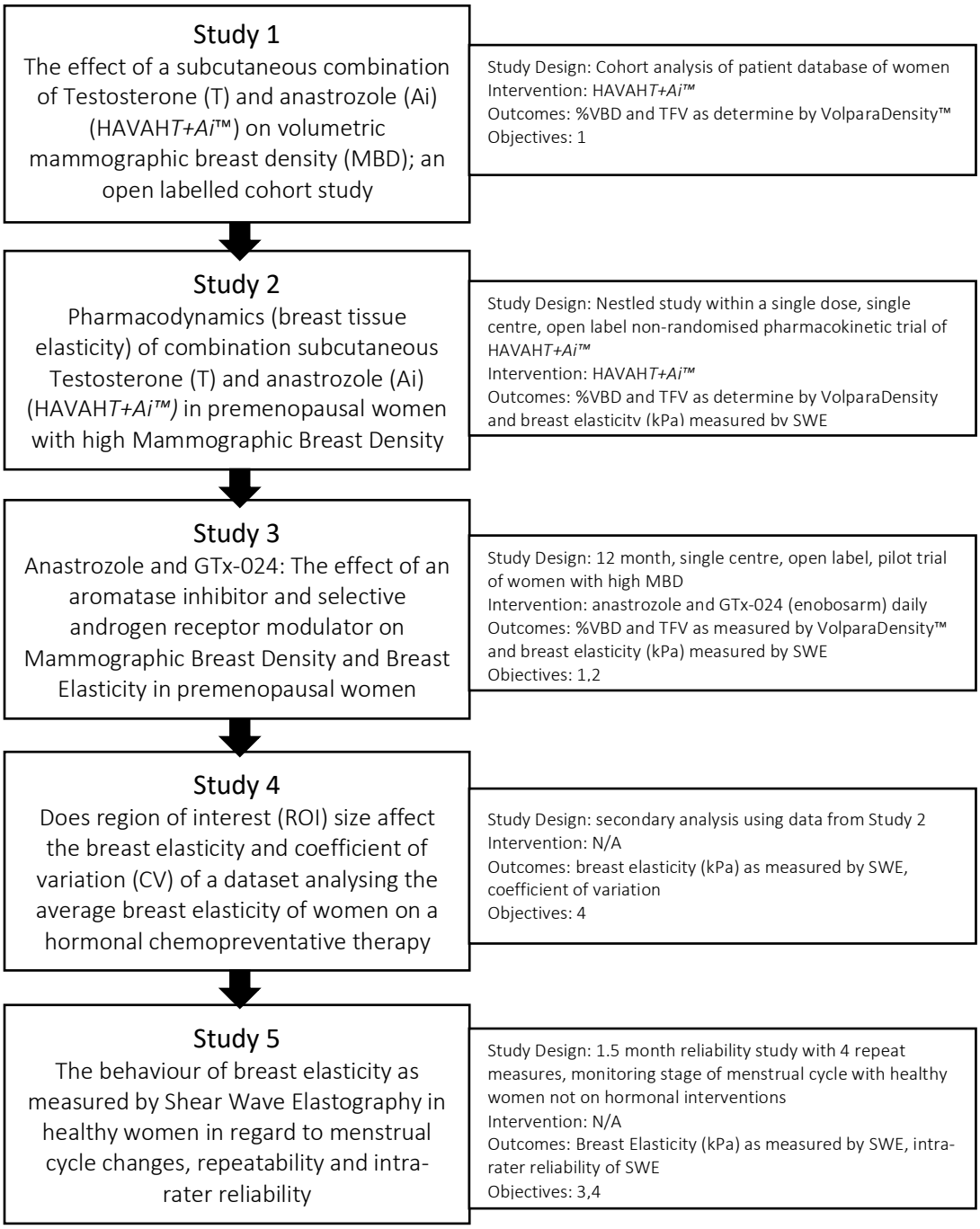

Figure 2-1: Research plan and associated objectives

Chapter 3 The Effect of a Subcutaneous Combination of Testosterone (T) and Anastrozole (Ai) (HAVAH*T+Ai*™) on Volumetric Mammographic Breast Density (MBD); an Open Labelled Cohort Study.

3.1 Background

As reported in section 1.2, MBD is a major, independent risk factor for breast cancer and there are limited interventions that have shown to be efficacious at reducing MBD, particularly in a premenopausal cohort as Ai's are contraindicated in this population. The overall primary aim of this thesis is to determine if breast elasticity can be used as a biomarker for MBD, especially in clinical trials due to its hypothesised sensitivity and clinical utility. In order to determine if the breast elasticity changes are correlating with the changes in MBD, it needs to be established that the hormonal interventions we are using in the subsequent clinical trials in this thesis are effective are reducing MBD.

This chapter is an analysis of clinical practice records of women who have been given the investigational product of HAVAH*T+Ai*™. Six hundred fifty-two women who attended Wellend Health Pty Ltd, Adelaide, South Australia, received HAVAH*T+Ai*™ as a subcutaneous implant every three to four months, were evaluated for MBD changes, as determined by VolparaDensity™ analysis software. One hundred forty-two of these women had two or more mammograms within the analysis, and a restricted analysis set (RAS) of 89 of these women were compared with a matched cohort 65 women undergoing mammographic screening for high risk of breast cancer but did not receive any hormonal therapy.

3.2 Objectives

The primary objective of this cohort analysis is to determine whether the administered HAVAH*T+Ai*™ therapy reduces MBD and whether this correlates with dosage and duration of therapy.

3.3 Publication Manuscript

The statement of authorship for the following publication manuscript is presented in Appendix 1.

The Effect of a Subcutaneous Combination of Testosterone (T) and Anastrozole (Ai) (HAVAHT+Ai™) on VolparaDensity™ Automated Volumetric Mammographic Breast Density (MBD); an Open Labelled Analysis of Clinical Practice Records.

1st author: Daniella Dougherty, The University of Adelaide
2nd author: Suzanne B Good, Wellend Health Pty Ltd
4th author: Professor Paul Rolan, The University of Adelaide
5th author: Nicholas J Birrell, HAVAH Therapeutics Pty Ltd; Wellend Health Pty Ltd
6th author: Dr Stephen Birrell, Havah Therapeutics Pty Ltd; Wellend Health Pty Ltd
sb@havahtx.com

All correspondence will be through Stephen Birrell
Email: sb@havahtx.com
Mobile Contact: (+61) 419 804 741

Abstract

Background

Mammographic breast density (MBD) is a modifiable risk factor for the development of breast cancer. An alteration in the oestrogen/androgen (E/A) ratio in favour of an androgenic environment may reduce MBD. It is hypothesised that HAVAH*T+Ai*™ may cause this favourable alteration. This large open-label cohort study evaluates the effect of HAVAH*T+Ai™* on MBD.

Method

Women who received HAVAH*T+Ai™* subcutaneous implant had their percentage volumetric breast density (%VBD) and absolute fibroglandular volume (FGV) measured by VolparaDensity™. Mixed model analyses were used to examine the drug dose relationship with %VBD and FGV. A restricted analysis set (RAS) of 89 women were compared with a matched control cohort of 65 women, their change from baseline in %VBD and FGV were analysed using a mixed model approach. Safety and tolerability data were collected and analysed.

Results

142 women were included in the analysis. Larger decreases in MBD were observed with accumulated testosterone (T) dosing of over 500mg compared with patients with <500mg. Change from baseline in %VBD by cumulative T dose, the largest reductions were -2.26% (95% CI -4.23% to -0.29%; p=0.00251) to -2.80% (95% CI -4.66% to -0.95%; p=0.0035) for the 500 – 700mg and 700mg+ strata, respectively. For change in baseline in FGV a cumulative T dose of 700mg+ demonstrated a reduction of -22cm^3 (95% CI -39.48 to -4.51; p=0.0142) and -36.21cm^3 (95% CI -59,71 to -12.71; p=0.0029), at years 2 and 3 respectively. No significant reductions in %VBD or FGV were observed in the control group. The treatment was well tolerated.

Conclusion

A cumulative dose of greater than 700mg of T and 30mg of Ai over 2 to 3 years, achieved a similar reduction in MBD as has been demonstrated with tamoxifen with better tolerability.

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women (Bray, Ferlay et al. 2018). Worldwide, there were approximately 2.1 million newly diagnosed female breast cancer cases in 2018, accounting for almost 1 in 4 cancer cases (Bray, Ferlay et al. 2018). In 2020, it has been estimated that there will be 279,100 new breast cancer cases in the United States alone (Siegel, Miller et al. 2020). The initiation and promotion of most, if not all, breast cancers are highly dependent on female reproductive hormones. Thus, manipulation of these hormonal pathways has been used to improve therapeutic outcome since the 1890s. Subsequently, anti-oestrogen therapies used for the treatment of breast cancer were shown to reduce the incidence of new breast cancers in the contralateral breast (Group 2005, Group 2011). Oral tamoxifen is the only widely accepted intervention registered for chemoprevention of breast cancer. However, tamoxifen has both agonistic and antagonistic impact on tissue-specific estrogenic effects, which results in significant adverse effects and contributes to a poor compliance rate. Approximately just 4% of women at increased risk of developing breast cancer utilise tamoxifen intervention (Ropka, Keim et al. 2010). Other treatments which appear to reduce the incidence of breast cancer, but have not achieved registration for this indication, include raloxifene, anastrozole and retinoids. Thus, there is an urgent need for new hormonal strategies to make

chemoprevention a more attractive option for women at high risk of developing breast cancer.

Mammographic breast density (MBD) is the ratio of glandular/stromal to fatty tissue within the breast and is a major modifiable risk factor for breast cancer. In a meta-analysis of 42 studies, McCormack and dos Santos Silva (2006) found that MBD was strongly associated with an individual's breast cancer risk, and MBD has been proposed as a potential surrogate endpoint of the efficacy of hormonal interventions (Shawky, Martin et al. 2016). It has been demonstrated that accumulative exposure of the breast to high MBD tissue is directly correlated with breast cancer risk (Cuzick 2003, Boyd, Berman et al. 2018), one prevention trial has demonstrated that reducing MBD can lead to a decrease in breast cancer risk. Cuzick, Warwick et al. (2011) reported that women treated with tamoxifen who had more than 10% absolute reduction in breast density (as visually assessed by reporting total breast area composed of dense tissue on an 0-100% scale) experienced a 63% decrease in breast cancer risk, as compared to no change in breast cancer risk in the placebo group and the women who did not respond to tamoxifen. Furthermore, many studies have evaluated the influence of tamoxifen associated MBD declines on breast cancer outcomes in the adjuvant setting (Li, Humphreys et al. 2013, Nyante, Sherman et al. 2015, Mullooly, Pfeiffer et al. 2016, Shawky, Martin et al. 2016). In particular, Li, Humphreys et al. (2013) reported, after a 15 year follow up, that women with breast cancer treated with tamoxifen as adjuvant therapy who experienced a reduction of more than a 20% relative reduction in absolute dense area in cm^3 had a 50% reduction in their breast cancer mortality, again compared to women who didn't respond to tamoxifen.

Therefore, reducing MBD in women at high risk of breast cancer may be of considerable benefit. There is usually a substantial reduction in MBD following menopause (Boyd, Martin et al. 2002) and this reduction correlates with the alteration in the oestrogen/androgen (E/A) ratio. The breast responds to this alteration in the E/A ratio (McNally and Stein 2017), such that when there is a shift towards a more androgenic environment, there is a substantial reduction in MBD. Therefore, it is hypothesised that pharmacologically enhancing this natural trend towards a more androgenic environment will result in a greater reduction in MBD, and this can be an effective way of lowering breast cancer risk.

A subcutaneous implant (HAVAH*T+Ai™)* containing testosterone (T) (80mg to 120mg), and the aromatase inhibitor (Ai) anastrozole in the dose range of 2 to 8mg, has been used in clinical practice as a custom pharmaceutical in Australia and the United States (Birrell, Butler et al. 2007, Glaser 2010). Combining T and anastrozole was initially used as a non-estrogenic treatment for the management of anastrozole-induced arthralgia and menopausal symptoms in women with breast cancer (Birrell and Tilley 2009, Glaser, York et al. 2014). The observation was made that this combination significantly reduced breast pain, which is closely linked to MBD (Birrell and Tilley 2009). As high MBD tissue has been demonstrated to have very high levels of aromatase (Vachon, Sasano et al. 2011), which results in high levels of tissue estradiol (even in the premenopausal breast) (Dabrosin 2005), the authors hypothesised that combining a pharmacological dose of T with an ultra-low dose of anastrozole would be adequate to shift the E/A ratio towards an androgenic environment. Subsequently higher levels of T would be made available for the 5α-reductase shift to the potent androgen 5α- dihydrotestosterone (5α-DHT). This intra tissue dynamic, would substantially change the tissue environment from an estrogenic to an androgenic environment milieu and drive down MBD.

This study aimed at utilising a subcutaneous combination of a pharmacological dose of T and a very low dose of anastrozole in a subcutaneous pellet to evaluate the impact of this therapy on MBD in addition to safety and acceptability of this treatment.

Method

This study was a single centre cohort study based at Wellend Health Pty Ltd, Toorak Gardens, South Australia. Patients were referred to the Wellend Health Pty Ltd for either high MBD, the management of menopausal symptoms, the treatment of breast pain or a combination of these factors. All patients gave written consent for their clinical data to be used for research.

All patients in the study cohort received a combination of T and anastrozole implants (HAVAH*T+Ai*™). The formulation consisted of a compressed implant containing crystalline T, anastrozole and magnesium stearate. A T dose of approximately 1mg per kilogram along with 2mg-8mg of anastrozole per subcutaneous implant was used in the first dosing. Dosing was

altered in subsequent implants depending on either symptomatic response or lack of breast tissue response.

Patients were started on the therapy following baseline evaluation consisting of medical history, mammography and biochemical and haematological testing, including reproductive hormone levels. The patients underwent repeat blood evaluations at four weeks, and 12 weeks after initiation of treatment, the included serum T and Sex Hormone Binding Globulin (SHBG) to allow the generation of Free Androgen Index (FAI). A clinical review occurred at six weeks, consisting of clinical examination and reporting of adverse events (AEs). The MedDRA coding dictionary (version 20.0) was used to standardise events named into preferred terms (PT) and place within system organ class (SOC). A new pellet was inserted at three to four months. This clinical process was repeated after each dosing, for the duration of each patient's therapy.

The primary efficacy measurement for patients treated for high MBD was a reduction in MBD. During the treatment period patients generally had a mammogram on an annual basis. Measurements of MBD were undertaken utilising automated MBD analysis software (VolparaDensity™) generating the following variables: percentage volumetric breast density (%VBD), the absolute volume of fibroglandular tissue in cm^3 (FGV), the volume of both breasts and BI-RADs score. As mammograms were not scheduled for specific time-points, a visiting windowing system was applied to the data in order to provide a specific data value to a specific time point. The windows were as follows; six months (180 days after first HAVAH*T+Ai*™ implant +/- 60 days), one year (365 days after first HAVAH*T+Ai*™ implant +/- 180 days), two years (730 days after first HAVAH*T+Ai*™ implant +/- 180 days), three years (1095 days after first HAVAH*T+Ai*™ implant +/- 180 days) and four years (1460 days after first HAVAH*T+Ai*™ implant +/- 180 days). Mammography variables were compared with the date of the first HAVAH*T+Ai*™ implant. If more than one mammogram was taken within a window, the results closest to the mid-point of the window were used. No windows were applied to the baseline value, that is, the baseline value could occur at any point prior to first HAVAH*T+Ai*™ implant date (the average number of days prior to the first implant for the baseline mammogram was 60 days).

In addition to the treatment group, mammographic data (%VBD and FGV) from an aged-matched control cohort of perimenopausal women at high risk of breast cancer who did not receive HAVAH*T+Ai*™ treatment were obtained from Wellend Health Pty Ltd clinic records. These women were not treated with HAVAH*T+Ai*™ due to personal preference as it is an experimental therapy and not covered by the Australian Governments Pharmaceutical Benefits Scheme. Each of the 65 women had data from two sequential breast cancer screening mammograms. A group of participants that had a mammogram both prior to and after HAVAH*T+Ai*™ implant were identified for comparison against the control cohort. This group was referred to as the restricted analysis set (RAS).

Statistical Analysis

Excel (Microsoft, USA) was used to collate and tabulate the data. SAS® for Windows Version 9.3 (SAS Institute) was used for the statistical analysis. To examine the drug dose relationship with the %VBD response, the change from baseline MBD was used as the outcome measure in a mixed model analysis using SAS PROC Mixed. This procedure allows for the potential of individual patients to contribute more than one mammogram following commencement of HAVAH*T+Ai*™ treatment (repeated measures analysis), as well as examining different covariance patterns amongst the data. From the data set provided for analysis, the following independent (explanatory) variables were used to examine their impact on the change in MBD: days since first implant, baseline %VBD MBD measurement (the value closest to, but not later than, the first HAVAH*T+Ai*™ implant), cumulative testosterone dose (mg) across the entire study (stratified into <500mg, 500mg to <700mg, and 700mg+), cumulative anastrozole dose (mg) across the entire study (as a continuous covariate), age (in years) at first implant, machine type (GE Healthcare or Hologic Inc mammography machine), the radiation dose at the mammogram, compression pressure at the mammogram, history of breast cancer (yes or no), the interaction term between days since the first implant and cumulative dose of testosterone strata. The last interaction term listed above allows for the fitting of different slopes to each of the T dose strata to see if any potential differences exist across the strata. In addition to the analysis conducted using %VBD MBD measurements, a complementary analysis was undertaken using a similar model, with the change from baseline FGV as the dependent variable. The only other change to the list of dependent variables was to replace baseline %VBD with absolute baseline FGV.

With the control cohort and RAS group, the 'change from baseline' in %VBD and in FGV were analysed using a mixed model approach, modelled controlling for the following items: age (for control cohort, age at first recorded mammogram and for RAS age at first HAVAH*T+Ai*™ implant), baseline %VBD/FGV (for control cohort, %VBD/FGV at first mammogram and for RAS, %VBD/FGV at mammogram closest to, but not after, first HAVAH*T+Ai*™ implant) and reference day (for control cohort, number of days between first and second mammogram and for RAS, number of days between first HAVAH*T+Ai*™ implant and follow up mammogram(s)).

Results

In total, HAVAH*T+Ai*™ dosing information from 652 women was provided for analysis. 142 patients had both pre-intervention baseline and subsequent mammograms. 65 patients were included in the control cohort with an average age of 49.6 (SD 7.24) years. 89 patients were included in the RAS group, with an average age of 51.3 (SD 6.89) years. Demographic and baseline summary information is provided in Table 3-1. All 652 patients were female, with an average age at the time of first HAVAH*T+Ai*™ implant of 52 years (range 23 to 79 years).

Table 3-1: Participant Characteristics

Characteristics	All Patients		Restricted Analysis Set		Control Cohort	
Age at first implant – Years (SD)	n = 652	52.3 (7.66)	n = 142	51.7 (7.26)	n = 65	49.6 (7.24)
Pre-implant %VBD - % (SD)		13.89 (7.89)		16.14 (8.14)		
Pre-implant FGV – cm^3 (SD)		156.26 (88.16)		170.77 (92.34)		
Indications						
Reduce BC risk (%)	n = 89	13.7%	n = 40	28.2%		
Hormonal Dysfunction	n = 334	51.2%	n = 43	30.3%		
Both	n = 177	27.1%	n = 58	40.8%		
Breast Cancer History						
Yes	n = 560	85.9%	n = 124	87.3%		
No	n = 90	13.8%	n = 18	12.7%		
Invasive Type BC						
Yes	n = 6	0.9%	n = 0	0.00%		
In-situ type BC						
Yes	n = 14	2.1%	n = 3	2.1%		
Reason for Stopping						
Ongoing Treatment	n = 365	56.0%	n = 107	75.4%		
Experienced adverse effects	n = 14	2.1%	n = 2	1.4%		
Cost of treatment	n = 30	4.6%	n = 3	2.1%		

Table 3-1 continued

Subject decided to cease HAVAH*T+Ai™* treatment	n = 46	7.1%	n = 7	4.9%		
Subject felt HAVAH*T+Ai™* treatment was not working	n = 28	4.3%	n = 5	3.5%		
Unexplained	n = 123	18.9%	n = 9	6.3%		
Doctor advised HAVAH*T+Ai™* treatment is no longer required	n = 16	2.5%	n = 4	2.8%		
Doctor advised HAVAH*T+Ai™* treatment is complete	n = 25	3.8%	n = 5	3.5%		
Subject was relocating	n = 4	0.6%	n = 0	0.0%		
Concomitant Medications						
Oestrogen-based Concomitant Medications Used						
No	n = 430	66.0%	n = 100	70.4%		
Yes	n = 222	34.9%	n = 42	29.6%		
Oral Concomitant Medications						
Yes	n = 10	1.5%	n = 2	1.4%		
Topical Concomitant Medications						
Yes	n = 192	29.4%	n = 34	23.9%		
Subcutaneous Concomitant Medications						
Yes	n = 13	2.0%	n = 4	2.8%		
Intravaginal concomitant medications	n = 52	8.0%	n = 12	8.5		

Measurement of Efficacy

Table 3-2 provides descriptive statistics for the change from pre-implant (baseline) mammography results. Although these values are unadjusted for other potential factors, both %VBD and FGV show a reduction following intervention with HAVAH*T+Ai*™ therapy. No data with a baseline pair was available for the 4-year post-implant window.

Table 3-2: Absolute change from baseline (pre HAVAHT+Ai™ implant) MBD measured by %VBD

Time-point	Summary Statistics	Value
6-month Window	n	12
	Mean (SD)	-0.56% (3.02)
1-year Window	n	94
	Mean (SD)	-1.77% (3.57)
2-year Window	n	48
	Mean (SD)	-1.44% (2.41)
3-year Window	n	13
	Mean (SD)	-2.02% (3.91)

Drug Dose and Relationship to %VBD Response

To examine more closely, the impact of HAVAH*T+Ai*™ intervention on MBD as measured by %VBD, the subsets of patients who had mammograms both before and after the commencement of treatment were considered. The mixed model estimates for change from baseline in %VBD are shown in Table 3-3. Statistically significant findings were noted for days since first HAVAH*T+Ai*™ implant and the interaction between days since first HAVAH*T+Ai*™ implant and cumulative testosterone strata. Specifically, larger decreases in %VBD were noted over time for patients with accumulated testosterone dosing of over 500mg compared with patients with <500mg. Baseline %VBD was also statistically significant, with higher baseline scores having larger observed changes (reduction in %VBD).

Efficacy of HAVAH*T+Ai*™ in the Reduction of MBD as Measured by %VBD and FGV

Table 3-3 presents the least square mean estimate of the change from baseline in %VBD, by cumulative T strata and time since the first implant. The largest reductions from baseline in MBD observed were for the 500-700mg strata, -1.69% (95% CI -3.01% to -0.38%; p=0.0121) at 2 years and -2.26% (95% CI -4.23% to -0.29%; p=0.0251) at 3 years and the 700+mg cumulative T strata with a reduction of -1.87% (95% CI -3.62 to -0.12; p=0.032) at 1 year, -2.36% (95% CI -3.88% to -0.79%; p=0.0034) at 2 years and -2.80 (95% CI -4.66% to -0.95%; p=0.0035) at 3 years. Table 3-4 presents the least square mean estimates of the change from baseline in FGV, by cumulative T strata and time since the first implant. The only values of significance were those in the 700+mg cumulative T strata with reductions of -22.00cm^3 (95% CI -39.48cm^3 to -4.51cm^3; p=0.0142) at 2 years and -36.21cm^3 (95% CI -59.71cm^3 to 12.71cm^3; p=0.0029) at 3 years.

Table 3-3: Least square change from baseline in MBD, by cumulative testosterone dose and time since first implant

Cumulative Testosterone Dose Group	Time Since First Implant	Least Square Mean Estimate	Lower 95% CI	Upper 95% CI	p-value
<500mg	1 year	-1.6085	-2.8628	-0.3542	0.0123
<500mg	2 years	-1.2130	-2.6838	0.2579	0.1053
<500mg	3 years	-0.8174	-2.8829	1.2480	0.4349
500 to <700mg	1 year	-1.1249	-2.4165	0.1668	0.0872
500 to <700mg	2 years	-1.6916	-3.0057	-0.3775	0.0121
500 to <700mg	3 years	-2.2584	-4.2275	-0.2893	0.0251
700+mg	1 year	-1.8688	-3.6155	-0.1222	0.0362
700+mg	2 years	-2.3358	-3.8817	-0.7899	0.0034
700+mg	3 years	-2.8028	-4.6566	-0.9490	0.0035

Table 3-4: Least square estimated change from baseline in FGV, by cumulative testosterone dose and time since first implant

Cumulative Testosterone Dose Group	Time Since First Implant	Least Square Mean Estimate	Lower 95% CI	Upper 95% CI	p-value
<500mg	1 year	-12.6001	-25.7233	0.5230	0.0597
<500mg	2 years	-11.2073	-28.3175	5.9029	0.1975
<500mg	3 years	-9.8144	-35.6603	16.0314	0.4537
500 to <700mg	1 year	0.2747	-12.2756	12.8251	0.9652
500 to <700mg	2 years	-4.1993	-19.8209	11.4223	0.5950
500 to <700mg	3 years	-8.6734	-34.0633	16.7166	0.4985
700+mg	1 year	-7.7803	-26.2516	10.6910	0.4052
700+mg	2 years	-21.9964	-39.4828	-4.5101	0.0142
700+mg	3 years	-36.2126	-59.7196	-12.7056	0.0029

Laboratory Tests

The prevalence of laboratory values out of the normal range is presented in Figure 3-1. Only patients with a pre-HAVAH*T+Ai*™ lab assessment are included in the figure to gauge the change from baseline values.

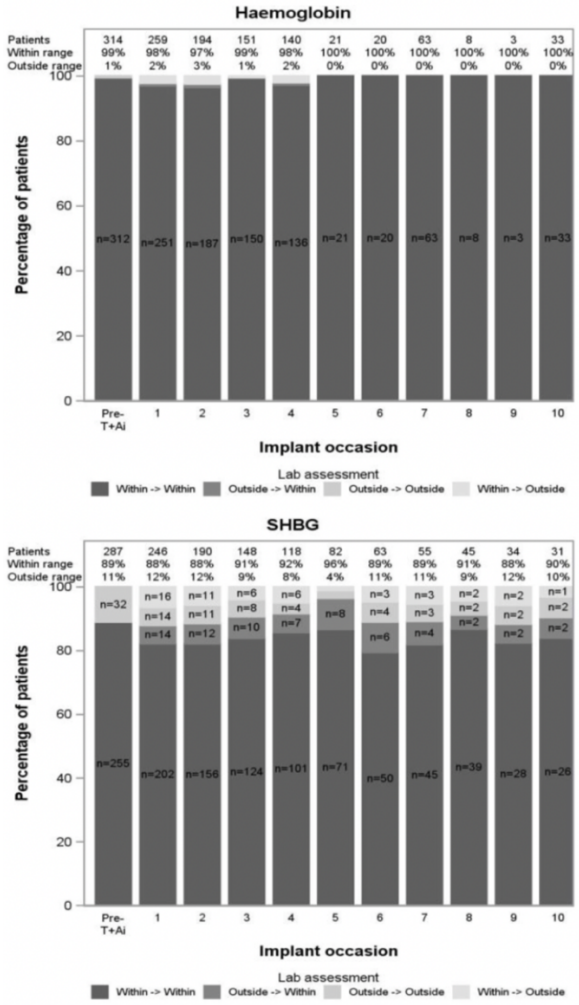

Figure 3-1: Prevalence of lab values out of normal range

Out of range FAI values were noted for 29% of patient's pre-HAVAH*T+Ai*™ (with denominator n= 279). The out of range FAI values increased to 78% of patients following the first implant with HAVAH*T+Ai*™ and then increased steadily over time, with over 90% of patients assessed (n= 54) at implant occasion 6 having values out of range. Out of range SHBG values were noted for 11% of patients pre-HAVAH*T+Ai*™ (with denominator n= 287) with the prevalence of out-of-range values remained relatively constant over the course of subsequent implants. Out of range testosterone values were noted for 9% of patient's pre-HAVAH*T+Ai*™ (with denominator n= 303). The prevalence of out of range values remained relatively constant over the course of subsequent implants with HAVAH*T+Ai*™.

Adverse Events

The most prevalent AEs were those seen in the skin and subcutaneous conditions SOC. Within this SOC, 58 (9%) patients reported hirsutism and 37 (6%) reported acne; this did not result in early termination of therapy. Other events with a prevalence of 4% or more included fatigue

(n=37 patients 6%), malaise (n=27 patients, 4%), weight increase (n=27 patients, 4%) and hot flushes (n=76 patients, 12%). Treatment-related AEs reported by five or more patients are displayed in Table 3-5. No deaths or serious adverse events that were related to HAVAH*T+Ai*™ occurred during the study time frame.

Table 3-5: Treatment related adverse events experienced by at least two patients, by average testosterone dose per implant

MedDRA Preferred Term	<80mg (n=182) S (%) E	80 to <90mg (n=273) S (%) E	90 to <100mg (n=106) S (%) E	100mg+ (n=71) S (%) E	All patients (n=652) S (%) E
Subjects with at least one AE	8 (4.4%) 19	10 (3.7%) 31	7 (6.6%) 15	4 (5.6%) 7	29 (4.4%) 72
Skin and subcutaneous tissue disorders	6 (3.3%) 8	9 (3.3%) 12	6 (5.7%) 7	3 (4.2%) 3	24 (3.7%) 30
Hirsutism	3 (1.6%) 3	5 (1.8%) 5	2 (1.9%) 2	1 (1.4%) 1	11 (1.7%) 11
Acne	4 (2.2%) 4	2 (0.7%) 2	3 (2.8%) 3	0 (0.0%) 0	9 (1.4) 9
Alopecia	0 (0.0%) 0	4 (1.5%) 4	1 (0.9%) 1	2 (2.8%) 2	7 (1.1%) 7
Psychiatric disorders	3 (1.6%) 3	3 (1.1%) 5	1 (0.9%) 3	1 (1.4%) 1	8 (1.2%) 12
Anxiety	2 (1.1%) 2	0 (0.0%)	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
Depression	0 (0.0%) 0	2 (0.7%) 2	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
Mood Swings	1 (0.5%) 1	1 (0.4%) 1	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
Gastrointestinal disorders	2 (1.1%) 3	2 (0.7%) 2	0 (0.0%) 0	0 (0.0%) 0	4 (0.6%) 5
Nausea	1 (0.5%) 1	1 (0.4%) 1	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
General disorders and administration site conditions	0 (0.0%) 0	2 (0.7%) 2	2 (1.9%) 2	0 (0.0%) 0	4 (0.6%) 4
Feeling abnormal	0 (0.0%) 0	1 (0.4%) 1	1 (0.9%) 1	0 (0.0%) 0	2 (0.3%) 2
Investigations	0 (0.0%) 0	3 (1.1%) 3	0 (0.0%) 0	1 (1.4%) 1	4 (0.6%) 4
Blood pressure increased	0 (0.0%) 0	2 (0.7%) 2	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
Weight increased	0 (0.0%) 0	1 (0.4%) 1	0 (0.0%) 0	1 (1.4%) 1	2 (0.3%) 2
Injury, poisoning and procedural complications [a]	0 (0.0%) 0	1 (0.4%) 1	2 (1.9%) 2	0 (0.0%) 0	3 (0.5%) 3
Nervous system disorders	1 (0.5%) 1	2 (0.7%) 2	0 (0.0%) 0	0 (0.0%) 0	3 (0.5%) 3
Vascular disorders	1 (0.5%) 1	1 (04%) 1	1 (0.9%) 1	0 (0.0%) 0	3 (0.5%) 3
Hot flush	1 (0.5%) 1	1 (0.4%) 1	1 (0.9%) 1	0 (0.0%) 0	3 (0.5%) 3
Reproductive system and breast disorders [a]	1 (0.5%) 1	1 (0.4%) 1	0 (0.0%) 0	0 (0.0%) 0	2 (0.3%) 2
Respiratory, thoracic and mediastinal disorders [a]	1 (0.5%) 1	0 (0.0%) 0	0 (0.0%) 0	1 (1.4%) 1	2 (0.3%) 2

[a] These are represented only at SOC level. There were no specific preferred terms within the SOC that occurred in two or more patients.
S (%) E – the number of patients with at least one event (percentage of patients with N as defined in the column heading), followed by number of events.

Control Cohort and Restricted Analysis Set

The results for the estimated change in %VBD and FGV after 2 years are presented in Table 3-6. For %VBD, after adjusting for age and baseline values, control cohort patients on average had an increase of 0.60% after two years, compared with a decrease of -1.76% in the RAS group. These findings represented a difference between the two groups of -2.36%, which was

a statistically significant difference from zero (95% CI -3.56% to -1.15%). For FGV, after adjusting for age and baseline values, control cohort patients on average had an increase of 6.4cm^3 after two years, compared with a decrease of -11.6cm^3 in the RAS group. These FGV values represent a difference between the two groups of -18.0cm^3, which was a statistically significant difference from zero (95% CI -31.1cm^3 to -4.8 cm^3). Based on the limited demographic information available from the control cohort, the modelling suggests that the intervention of HAVAH*T+Ai*™ therapy has a significant effect on MBD outcome measures of %VBD and FGV compared to the control cohort.

Table 3-6: The estimated change in %VBD and FGV after 2 years in Control Cohort and RAS group

	Estimated Change in %VBD (95% CI) After 2 Years	**Estimated Change in FGV (cm^3) (95%CI) After 2 Years**
Control Cohort	0.60 (-0.27, 1.47)	6.4 (-2.8, 15.5)
RAS	-1.76 (-2.52, -1.00)	-11.6 (-20.9, -2.3)
Difference (RAS – Control)	-2.36 (-3.56, -1.15)	-18.0 (-31.1, -4.8)

Discussion

It has been demonstrated that the maximal reduction in MBD with tamoxifen is seen at 2 years following initiation of therapy (Cuzick, Warwick et al. 2011). Unlike cancer therapy, where dose maximisation frequently is traded off against AEs, prevention therapy needs to be the lowest dose possible to affect a response and ensure that the side effect profile provides a clear benefit over the AE relationship. This patient cohort analysis using VolparaDensity™ obtained from 142 patients with mammograms taken both pre- and post-commencement of HAVAH*T+Ai*™ therapy showed there were statistically significant changes from baseline MBD measurements of both %VBD and FGV following intervention with HAVAH*T+Ai*™ therapy.

When MBD was measured by %VBD, patients with cumulative T dosing of 700mg+, the one-year post-commencement of therapy estimated change was -1.87%, the two-year post-commencement of therapy estimated change was -2.34%, and the three-year post commencement point of therapy had an estimated change of -2.82%. These figures result in a relative %VBD change of -11.6%, -14.5% and -17.5% respectively. For patients with cumulative T dosing of 500-<700mg+, the one-year post-commencement estimated change was -1.12%

(p=0.09), the two-year post-commencement estimated change was -1.69%, and the three-year post commencement of therapy estimated change was -2.26%. These results equated to a -6.9%, -10.5% and -14% relative %VBD decrease, respectively.

When the MBD variable of interest was FGV, the only values of significance were those in the 700mg+ cumulative T group, at 2 and 3 years post-first HAVAH*T+Ai™* implant. The absolute change from FGV estimates were -22cm^3 and -60cm^3 at 2 and 3 years, respectively. These results equate to a -12.9% and -35.1% relative decrease. Both results demonstrate clinically significant findings as per the previous research reported in the literature. As discussed in the introduction for this paper, Cuzick, Warwick et al. (2011) reported that a 10% reduction on a 0-100% scale is required for a 63% decrease in breast cancer risk, which was reached in the first year with 700mg+ T cumulative dosing and at the third year on 500-700mg+ T cumulative dosing. In addition, a 20% decrease in FGV has been reported in the literature to lead to a 50% reduction in breast cancer mortality which was accomplished at the third year with 700mg+ cumulative T dosing (Li, Humphreys et al. 2013). The model fitted also suggested that cumulative anastrozole dosing is a variable of interest when assessing change in MBD (p=0.06). Larger values of cumulative anastrozole doses were associated with larger changes from baseline (decrease from baseline) in MBD.

The other clinically meaningful benefit of HAVAH*T+Ai™* was that there was a favourable AE profile of the therapy. Factors that are known to be associated with oral T (elevated haematocrit, elevated liver enzymes, elevated adverse lipid profile) were not observed. There were some increases in androgenic effects such as hirsutism, but these were not enough to cause a cessation of therapy. Local AEs were infrequent and were not graded for severity, but most of those that were listed as site haemorrhage were bruising rather than overt bleeding.

This cohort patient analysis has exhibited findings that combination therapy of T and anastrozole (HAVAH*T+Ai™)* results in a statistically and clinically significant reduction of %VBD and FGV. To achieve the clinically significant level of MBD reduction that was seen in the IBIS-1 tamoxifen breast cancer prevention study (Cuzick, Warwick et al. 2011), a minimum cumulative dose inclusive of 700mg of T in combination with anastrozole needs to be given. Although dosing of less than 700mg of cumulative T exposure in combination with anastrozole was shown to affect significant changes in %VBD as a measure of MBD. FGV is a

more robust measurement of breast tissue response as it is the least likely of the measures to fluctuate with changes in BMI (Krishnan, Baglietto et al. 2017). %VBD needs to be corrected over time for the change in BMI, and this was not captured in this cohort analysis; this may be the reason for greater heterogeneity in the dose-response seen when measuring %VBD compared with FGV.

However, the most significant response in FGV and %VBD was when there was greater than 700mg of T given in combination with anastrozole over a two to three-year period. Larger values of cumulative anastrozole dose were associated with greater changes from baseline (decrease from baseline) in %VBD ($p=0.06$ when the significance level was set at $p=0.05$). It was demonstrated that the average anastrozole dose in this cohort of 2mg per implant was not adequate. Based on these observations, suggested dosing for reduction of MBD should include a mid-range dose of T (80mg per implant) and an upper-end range of anastrozole (4mg per implant). Therefore, a treatment regimen of 10 implants administered over 2.5 years, delivering a cumulative dose of 800mg of T in combination with 40mg of anastrozole should achieve the target clinical effort on MBD (in line with tamoxifen) while maintaining a low incidence of AEs and good patient tolerability.

Due to the nature of this research, there needs to be some consideration with the interpretation of these findings. Firstly, as this was an analysis of patient records the results may be affected by selection bias, the participants that are returning for repeat implant dosages might be the patients who responded well to the treatment in regard to decreasing MBD variables and the interventions side effect profile. Therefore, the decreasing %VBD and FGV values with the increasing cumulative T dosing may not represent the true results that would be present in the intended population. Furthermore, limited demographic information was documented for the control cohort; therefore, we were unable to determine if the baseline characteristics were significantly different from the intervention group. This discrepancy may lead to a different outcome occurring if the research was to be replicated. With the information presented, it would be feasible and beneficial to conduct further research of a more rigorous design, using the knowledge that HAVAH*T+Ai™* has signs of effect of being able to reduce %VBD and FGV in women at increased risk of breast cancer.

Overall, this patient cohort analysis demonstrated that the hormonal intervention of T and anastrozole (HAVAH*T+Ai™)* was efficacious at reducing MBD, with no suggestion of increasing AE prevalence with increasing T dose, in a perimenopausal patient population with high MBD. This analysis has produced the first report of the impact of combining a pharmacological dose of T with a low dose of anastrozole on MBD; these results suggest a larger study should be undertaken to confirm the efficacy and safety of this therapy as a chemopreventative intervention for breast cancer.

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Notes

Conflicts of interest: Stephen N. Birrell and Nicholas J. Birrell are both founders of HAVAH therapeutics, which has the proprietary rights to HAVAH*T+Ai™*

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3.4 Publication Summary

To date, clinical observations of subcutaneous testosterone and an anastrozole implant as a hormonal replacement have shown favourable results. Compared to previously conducted studies, the incidence of breast cancer in the treated women (142/100,000) is substantially lower than it is in SEER age-specific incidence rates (293/100,000), the placebo arm of the Women's Health Initiative (300/100,000), and the never users of hormonal therapy from the Million Women's Study (325/100,000) (Glaser and Dimitrakakis 2013). Glaser and Dimitrakakis (2013) originally stated that breast cancer is preventable by maintaining an androgenic environment concerning the testosterone/oestrogen ratio. It has previously been demonstrated that there is an association between hormone exposure, MBD, and future risk of breast cancer (Boyd, Melnichouk et al. 2011). This cohort analysis contributes to previous research by Glaser and Dimitrakakis (2013) demonstrating a potential association with HAVAH*T+Ai*™ therapy, the shift to an androgenic breast tissue environment, a reduction in MBD variables, and a possible reduction in breast cancer risk.

Although there is evidence to show the previously stated associations, the study did have some significant limitations that require further discussion. Firstly, the main limitation of this analysis is the study design itself. As it was an open-label uncontrolled record of clinical practice rather than a double-blind, randomised controlled trial (RCT), we were unable to establish a direct causal link between HAVAH*T+Ai*™ and the reduction of MBD and TFV in this patient cohort. However, the observations reported met some criteria of the Bradford Hill criteria for causality (Hill 1965), specifically temporality, biological gradient and plausibility as the reductions in mammography variables occurred after the commencement of HAVAH*T+Ai*™ therapy, showed greater reductions with increasing dose and aligned with the hypothesised changes based on the current body of evidence, so a causal link between the therapy and a reduction in MBD and TGV is likely. More rigorous research designs, such as the RCT mentioned above are required to be undertaken to determine if HAVAH*T+Ai*™ is the causal reason for the reduction in the mammography variables and the subsequent potential breast cancer reduction.

A further limitation of this study was that HAVAH*T+Ai*™ is currently being used for numerous indications such as menopausal symptom and breast pain management. Due to this reason,

within the cohort of 652 women, only 89 patients (14%) were given the therapeutic intervention for an indication of a reduction in MBD/breast cancer risk. As there was limited baseline MBD data, and we analysed the correlation between dosage and MBD, this could be a confounder as the patients in this cohort may have had a lower MBD at baseline. In saying this, the patients that had a baseline mammogram, although unadjusted for other potential factors, showed a reduction in %VBD and TFV MBD measurements, following the HAVAH*T+Ai*™ intervention irrespective of baseline MBD. Another limitation within this analysis was the limited data regarding the tolerance of HAVAH*T+Ai*™; 123 women were lost to follow up, 46 decided to cease therapy, and 28 felt that HAVAH*T+Ai*™ was not working. It is important that for future studies the drug tolerance is analysed and any reasoning for the cessation of the therapeutic intervention is understood and documented.

3.5 Conclusion

Regarding the overall aims and objectives of this analysis of clinical practice records, there is evidence that HAVAH*T+Ai*™ has signs of effect concerning reducing MBD in a patient cohort with measures high MBD. Future research needs to be conducted with more rigorous, statistically powered, placebo-controlled, double-blind RCT. Within these studies, there will also need to be a more specific inclusion and exclusion criteria which incorporates women with high MBD. This eligibility criterion will allow the study population to be an accurate representation of the population of interest. Also, any study of this nature will need to incorporate baseline measures and repeat measures of the mammography variables, in addition to safety and tolerability data. Utilising this study design will allow the effect size of the intervention on the MBD variables to be calculated and evidence to be presented regarding the safety and overall tolerability of the drug.

3.6 Future Research

The initial hypothesis was that breast elasticity might be a viable biomarker for MBD and the associated aims and objectives, as reported in Section 2, were trying to establish whether this is the case. The results from this study were produced so they could be applied to the subsequent studies within this thesis. The findings from this chapter demonstrate that HAVAH*T+Ai*™ was associated with a reduction in %VBD and TFV. The future studies within this

doctoral thesis can incorporate the breast elasticity outcome measure with the use of the SWE ultrasound machine. The breast elasticity can be measured at more frequent intervals than mammography. This study design will allow us to determine if breast elasticity also changes in response to this hormonal intervention, if these changes correlate with the changes occurring in the mammography variables and whether the breast elasticity is more sensitive to detect changes.

Chapter 4 Pharmacodynamics (breast tissue elasticity) of Combination Subcutaneous Testosterone and Anastrozole (HAVAH*T+Ai*™) in Premenopausal Women with High Mammographic Breast Density

4.1 Introduction

As reported in Chapter 3, HAVAH*T+Ai*™ has demonstrated the potential to be efficacious at reducing %VBD and TFV within a cohort of women with high MBD and therefore increased breast cancer risk. This study is the initial study within this research program for which we have incorporated the measure of breast elasticity to evaluate the response of the breast tissue to the investigational product. The study within this chapter is a sub-study analysis within a single-dose pharmacokinetic trial of HAVAH*T+Ai*™. As the results in Study 1 (Chapter 3) suggest, repeat dosages of HAVAH*T+Ai*™, which led to a cumulative testosterone dosage of 500 to 700mg+, were found to be the most efficacious for reducing MBD. Therefore, as this is a single-dose study, it is not hypothesised that MBD and its associated variables will change in response to the investigational product; however, there may be a short-term response seen in the breast elasticity.

As mentioned in Section 1.2.4, the breast is embryologically a modified sweat gland, and it responds like a sweat gland to any alteration to its E/A ratio (McNally and Stein 2017). When there is a shift in this ratio towards an androgenic tissue environment in the breast, there is a substantial change in MBD (as is seen following menopause) (Boyd, Martin et al. 2002). The only widely accepted intervention shown to achieve this effect in premenopausal women is oral tamoxifen. As a partial agonist of oestrogen, tamoxifen causes significant alterations in both breast and systemic oestrogenic stimulation; this systemic oestrogenic stimulation can result in significant treatment-related side effects that reduce the compliance with tamoxifen as a breast cancer chemopreventative agent (Peres 2014).

Within the breast, there are many enzymes that convert reproductive pro-hormones, including aromatase (Vachon, Sasano et al. 2011) and 5α-reductase (Suzuki, Miki et al. 2006). These enzymes convert testosterone to either E_2 or DHT, the latter being ten times more potent than testosterone as an androgenic agent. High MBD tissue has been shown to

contain very high levels of aromatase (Vachon, Sasano et al. 2011), resulting in enhanced intracrine production of oestrogen. Aromatisation of testosterone is important in both the pre-and postmenopausal breast (Dabrosin 2005).

By using HAVAH*T+Ai*™, we intend to utilise the overexpression of these enzymatic systems in high MBD breast tissue by treating with a pharmacological dose of testosterone combined with a low dose of an Ai, thus shifting the E/A ratio towards an androgenic tissue environment. This androgenic environment results from the Ai blocking the conversion of testosterone to oestradiol, thus increasing bioavailable testosterone in the breast tissue. In addition, higher serum testosterone results in more of this androgen being delivered to the breast. Ultimately, the consequence of these two actions is a high level of intra-mammary testosterone being made available for conversion to DHT and a reduction in intra-mammary E_2.

As previously mentioned in Section 1.5, within the tissue of high MBD, there is decreased activity of the extracellular matrix-degrading enzymes, MMPs and an overexpression of their specific inhibitors (TIMPs). The organisation of the extracellular matrix is likely to play a role in mediating the mechanical properties of tissues and may influence the elasticity of the tissue. Nilsson, Garvin et al. (2007) conducted research into the effect of oestrogen and tamoxifen on the secretion and activity of MMP-2 and MMP-9 and TIMP-1 and TIMP-2, finding that tamoxifen treatment induced a significant increase in MMP-2/MMP-9 activity ($P<0.001$ as compared to control cells) and that oestradiol significantly decreased MMP-2/MMP-9 activity ($P<0.05$ as compared to control cells) and this decrease was in part reversed by addition of tamoxifen to the oestradiol treatment ($P<0.01$). Nilsson, Garvin et al. (2007) also reported that tamoxifen significantly increased TIMP-1 levels ($P<0.05$ as compared to control) whereas oestradiol significantly lowered the amounts ($P<0.01$ as compared to control cells). We hypothesised that by using HAVAH*T+Ai*™ to decrease the oestradiol levels within the breast, there will be an increase in the extracellular matrix degrading proteins of MMP-2 and MMP-9 and an increase in the TIMP-1 and TIMP-2 tissue inhibitors, which would lead to greater extracellular matrix regulation. Therefore, breast tissue elasticity may change in response to the HAVAH*T+Ai*™ intervention, and this could be detected earlier than the global tissues as seen with mammography.

4.2 Objectives

This is the first study within this thesis that directly measures the effect of a chemopreventative agent (HAVAH*T+Ai*™), that has been shown to reduce MBD (as per Chapter 3), on breast tissue elasticity. This study evaluates the potential effect on HAVAH*T+Ai*™ on breast tissue elasticity, as measured by SWE and whether these changes correlate with changes (if occurring) in MBD, as measured by VolparaDensity™.

4.3 Method

This pharmacodynamic analysis was a sub-study study within a single-dose, single-centre, open-label non-randomised pharmacokinetic trial of HAVAH*T+Ai*™ in premenopausal women conducted at Wellend Health Pty Ltd, Toorak Gardens, South Australia. This trial was approved by the Bellberry Limited Human Research Ethics Committee (HREC) (approval number 2017-06-434). As the purpose of the trial was descriptive, a formal sample size calculation was not appropriate. A planned sample size of 12 participants was based on feasibility, and it was anticipated that sufficient information would be obtained to achieve the primary pharmacokinetic objective of the trial.

4.3.1 Patient Population

The trial population consisted of premenopausal women. Potential participants were those either referred by their medical practitioners or self-referred to the Wellend Health Pty Ltd clinic for HAVAH*T+Ai*™ therapy for the reduction of high MBD. Potential patients were screened for their eligibility to be included in the study during the 21 days prior to the scheduled dosing date. Participants were eligible for the study if the following criteria were met; premenopausal levels of follicular stimulating hormone, luteinizing hormone and oestradiol (FSH/LH/E2) according to the definition of “premenopausal range”, VolparaDensity™ volumetric breast density of ≥ 15.5% (combined average both breasts), aged between 33-55 years inclusive, body weight between 50 and 90kg inclusive, in good general health without clinically significant cardiac, respiratory, or psychiatric disease and a negative pregnancy test. Participants were excluded from the trial if there was the presence of breast cancer, had a previous or concomitant other malignancy (non-breast, other than skin) within the previous five years, diabetes mellitus or glucose intolerance, history of coronary artery

disease, existing testosterone, oestrogen and/or anastrozole treatment or systemic hormonal contraception. Participants who prematurely withdrew post-dosing were to be replaced to ensure that at least 11 participants completed the trial. Prior to dosing, the participants were confirmed to be in the luteal phase of their menstrual cycle, as based on progesterone levels of >4nmol/L.

4.3.2 Investigational Product

All participants received the same active treatment of HAVAH*T+Ai™,* a subcutaneous pellet with 80mg of testosterone and 4mg of anastrozole. This dosing regimen of HAVAH*T+Ai™* is appropriate for a population of the age and BMI set out in the inclusion criteria. The participants were advised to place a patch of EMLA local anaesthetic cream (a combination of lidocaine and prilocaine) at least one hour before the planned administration of the HAVAH*T+Ai™* at the site of the proposed insertion. The investigational product was administered to each participant by Dr Stephen Birrell, The Medical Director of Wellend Health Pty Ltd. Prior to administration, a standard sterile procedure was undertaken, and 5ml of xylocaine was injected with a 25-gauge needle on the right gluteal region midway between the greater tuberosity of the hip and the superior tuberosity of the pelvis. A 4mm incision was made through the skin, and a 4mm trochar was inserted at an angle of 45˚ into the subcutaneous tissue for a length of 5cm. The implant was inserted to the end of the trochar, after the withdrawal of the trochar, the wound was closed with a butterfly closure such as steri-strips and covered with a waterproof dressing. The pellet was inserted between 8 am and 10 am on the day of dosing.

4.3.3 Outcome Measures

All participants had a baseline mammogram conducted by Dr Jones and Partners Medical Imaging at Burnside Hospital. These mammography images were analysed by VolparaDensity™ software, which provided the variables of %VBD, TFV and total breast volume in cm^3 (TBV). The participants also consented for their 12-month annual follow-up mammogram to be analysed as part of this study.

Breast elasticity was measured with SWE and was conducted using the SuperSonic™ Imagine Aixplorer® ShearWave™ ultrasound (Aixplorer®, France) (Figure 4-1). A linear transducer head with ample ultrasound gel (Figure 4-2) was placed on the breast, parallel from the nipple, two centimetres away from the nipple in a diagonal direction (Figure 4-3).

Figure 4-1: SuperSonic™ Imagine Aixplorer® ShearWave™ Elastography ultrasound device

Figure 4-2: Linear transducer head with ultrasound gel

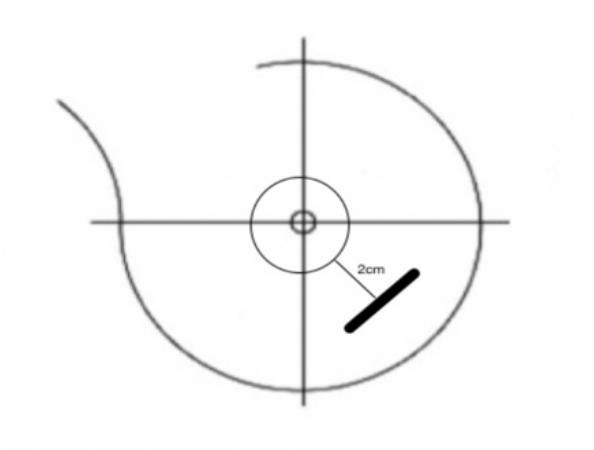

Figure 4-3: Diagram of the location and direction of the linear transducer head on the breast

The breast was visually divided into eight quadrants (Figure 4-4), in reference to Figure 4-4, number one is the right outer lower quadrant, number two is the right outer upper quadrant, three was the right inner upper quadrant, four is the right inner lower quadrant, five is the left inner lower quadrant, six is the left inner upper quadrant, seven is the left outer upper quadrant and eight is the left outer lower quadrant. Once the images were captured, they were analysed *post-hoc* on the SWE machine. To analyse the image and generate the elasticity data, a single or multiple pre-defined circular Q-Box™ were placed on the image (Figure 4-5), within this Q-Box™ a ROI was created and the minimum, maximum, mean and standard deviation (SD) of the tissue elasticity in kilopascals (kPa) and the depth of the tissue was calculated from this ROI. For this study, six 3mm (6 x 3mm) Q-Box™ were placed on the image (Figure 4-5).

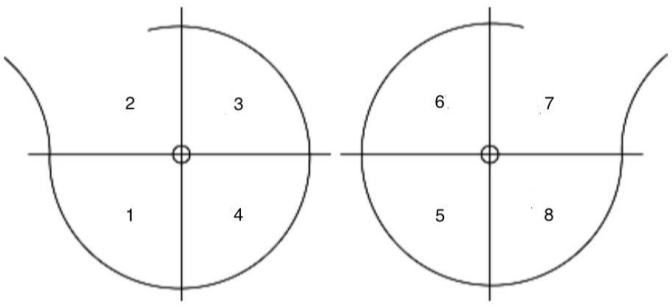

Figure 4-4: Image sequence of the breast quadrants used in shear wave elastography

The average of all six Q-Box™ per image were combined to determine the average elasticity of both breasts combined per participant. The Medical Director at Wellend Health Pty Ltd advised the method of using 6 x 3mm Q-Box™ as the best method for acquiring the elasticity

data and therefore was used within this study. Once the images had the Q-Box™ and elasticity measurements generated, a report was made with all the images and all the corresponding data which was exported from the machine. Breast elasticity was assessed at baseline, and on days 29, 57 and 85.

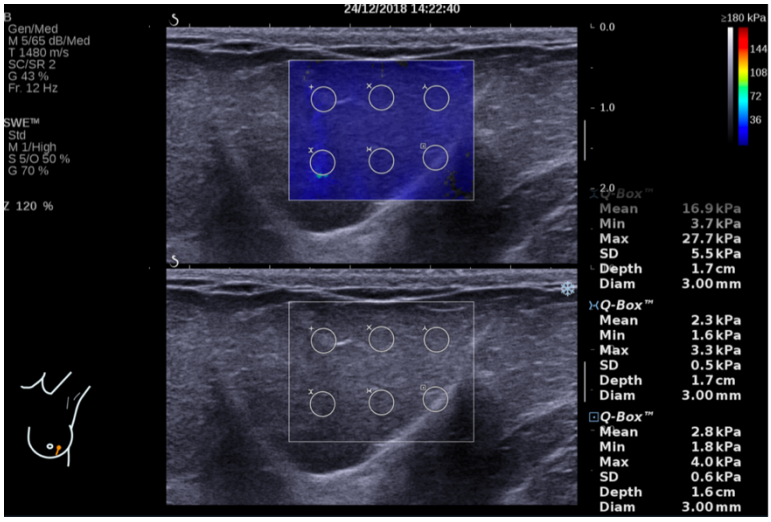

Figure 4-5: Shear wave ultrasound output with six 3mm Q-Box™ and the elasticity measurements

4.3.4 Statistical Analyses

All collected data was collated and tabulated using Microsoft Excel (Microsoft, USA). All statistical analyses were conducted using IBM SPSS version 25 (IBM, Amarok, USA). The statistical model chosen for each analysis is described in the corresponding result section.

4.4 Results

4.4.1 Patient Demographic

11 participant datasets were included in this analysis. The average age of the participants was 41.5 (SD 3.7) years; a full summary of the participant demographics is presented in Table 4-1. All participants had their data included in all the analyses.

Table 4-1: Participant characteristics

Demographic Parameters (Units)	Average (SD)
Age (years)	41.4 (3.7)
Weight (kg)	67.87 (10.27)
Height (m)	1.673 (0.066)
BMI (kg/m2)	24.14 (2.26)
	Number of Participants (%)
Race (white)	10 (90.9%)
Race (white/Asian)	1 (9.1%)
Ethnicity (not Hispanic or Latino)	11 (100%)

4.4.2 Changes in Breast Elasticity from Baseline to Month 3

The descriptive summary of the breast elasticity values and the change in breast elasticity are presented in Table 4-2.

Table 4-2: Mean Breast elasticity summary and change from baseline

Timepoint	Breast Elasticity (SD) in kPa	Change from Baseline (kPa)
Baseline	13.67 (7.89)	
Month 1	11.68 (5.58)	-1.99
Month 2	9.86 (5.63)	-3.80
Month 3	8.63 (3.96)	-5.04

Due to the small sample size of this study, a spaghetti plot was created to visualise the patterns for each study participant (Figure 4-6). This plot shows that there is significant variation within each individual dataset. The most common trajectory was a breast elasticity decrease from baseline, however there were two participants whose breast elasticity increased over the three weeks.

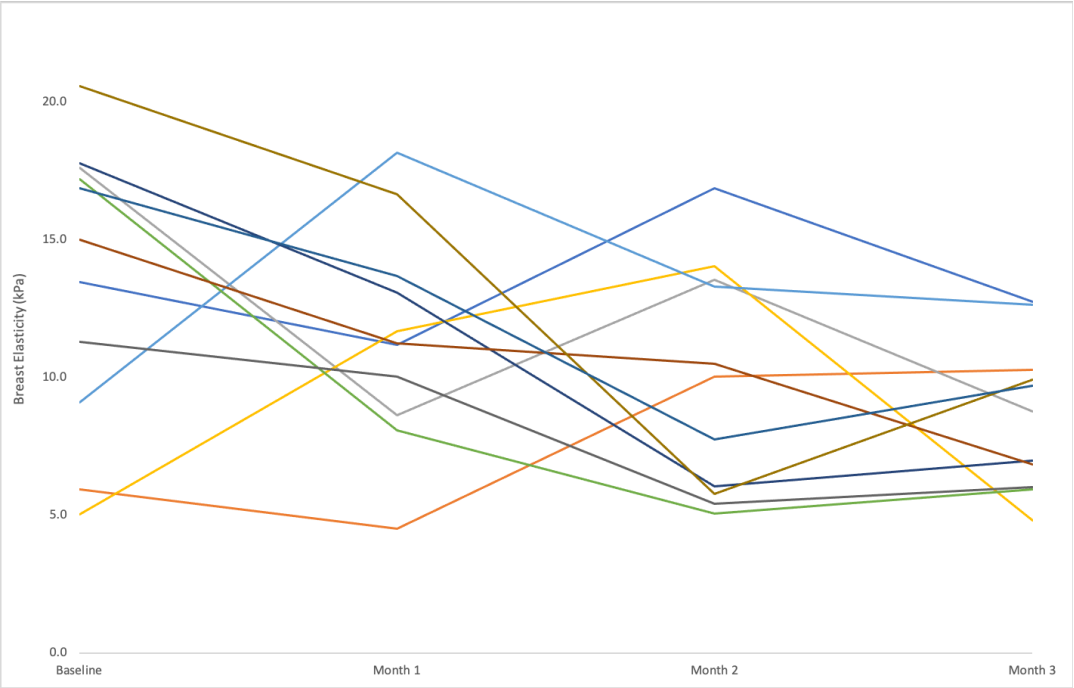

Figure 4-6: Breast elasticity for each individual participant, at each timepoint

Upon analysing the data, it was observed that there were outliers present in each data set. All of these data points were assessed and deemed to be true values and were left within the analysis. The original data were not normally distributed as determined by the Shapiro-Wilk test; for this reason, the Friedman test, a non-parametric statistical model, was used to analyse the data.

The results show that breast tissue elasticity was statistically significantly different at the different time points during the intervention $X^2(3) = 30.835$, $p<0.0005$. The median values of the breast tissue elasticity at each time point are presented in Table 4-3. Pairwise comparisons were performed with Bonferroni corrections for multiple comparisons. The breast elasticity was statistically significantly different between baseline and month 2 ($p=0.002$) and baseline and month 3 ($p<0.0005$). There were no statistically significant differences between baseline and month 1 ($p=1.000$).

Table 4-3: Median breast elasticity values

Timepoint	Median Breast Elasticity (kPa)	p-value (difference from baseline)
Baseline	13.10	
Month 1	10.80	1.000
Month 2	8.39	0.002
Month 3	7.98	<0.0005

4.4.3 Changes Between Percentage Volumetric Breast Density from Baseline to Month 12

Within the study protocol, the participants were scheduled to have a mammogram after 12-months. From this data, it enabled us to determine the effect of HAVAH*T+Ai*™ on %VBD across 12 months. The women all had differing dosages levels, due to the small sample sizes, the results will be provided as the overall changes and then descriptive statistics regarding the different dosage groups.

Upon looking at the data sets, the data were normally distributed, and there were no outliers present in either data set. The mean %VBD of the baseline data was 19.41% (SD 3.07), and the 12-month data was 18.08% (SD 4.91%). The results of a paired-samples T-Test showed that there was a statistically insignificant mean difference of -1.33% (95% -1.61% to 4.26%; p= 0.34). As mentioned, that treatment doses varied between participants; Table 4-5 shows the descriptive statistics of the %VBD changes as per the number of HAVAH*T+Ai*™ pellets inserted.

Table 4-4: Descriptive statistics of the %VBD changes as per the number of HAVAHT+Ai™ pellets

Dose (number of pellets)[a]	Number of Participants	Baseline Mean %VBD	12- Month Mean %VBD	Mean %VBD Absolute Change
1	2	18.4%	16.5%	-1.9%
2	1	17.5%	18.3%	0.8%
3	5	18.9%	16.36%	-2.54%
4	2	20.0%	21.2%	1.2%
5	1	24.7%	23.4%	-1.3%

[a] The number of pellets varied due to the participants having the choice of continuing therapy or discontinuing therapy once the 3-month study had reached completion

4.4.4 Changes in Total Fibroglandular Volume from Baseline to Month 12

The TFV (cm^3) is a mammography variable that calculates the volume of fibroglandular (or dense) tissue within the breast. The data of the baseline TFV was not normally distributed, and there was one outlier present in the baseline dataset, which was assessed and deemed to be a correct figure and was left in the analysis. As the data was not normally distributed, a non-parametric statistical model was chosen to analyse the data. Table 4-6 shows the mean descriptive statistics for the dose-specific changes.

The mean fibroglandular volume of the baseline dataset was 197.4cm^3 (SD 118.7cm^3) and 184.4cm^3 (SD 125.1cm^3) for the 12-month dataset, this was a mean difference of -13.02cm^3 (SD 29.55).

Table 4-5: Descriptive statistics of the TFV changes as per number of HAVAHT+Ai™ pellets

Dose (number of pellets)	Number of Participants	Mean Baseline (cm^3)	Mean 12-Month (cm^3)	Change (cm^3)
1	2	214.55	197	-17.55
2	1	196.3	254.4	58.1
3	5	137.32	107.24	-30.08
4	2	389.6	386.8	-2.8
5	1	80	69.8	-10.2

When using the non-parametric statistical models, the median values and median differences are the data used unless otherwise stated. The distribution of the differences between the two groups was not symmetrical in shape; for this reason, the Sign test was chosen as it does not require the assumption of symmetry within the differences.

Eleven participants were recruited into the pharmacokinetic study, and all 11 had a baseline and 12-month follow-up data recorded. All data are median values unless otherwise stated. In the analysis, when comparing the baseline and TFV at baseline and month 12; participants had a lower TFV at 12-months (126.90cm^3) compared to the baseline data (154.10cm^3) with a median difference of -22cm^3, this result had a p-value of 0.065. From the 11 participants, two had increased, and nine had decreased TFV, there were zero ties within the dataset.

4.4.5 Correlations Between Per Cent Volumetric Breast Density and Breast Elasticity

This section contains three different statistical analyses to determine if;

1. The baseline %VBD were correlated with the breast elasticity
2. The 12-month %VBD measures were correlated with the 3-month breast elasticity measures
3. The changes in %VBD were correlated with the change in elasticity from baseline to the final measures

For parametric data, the Pearson's correlation coefficient and for non-parametric data, the Spearman Rank Order correlation was used to determine if there was a relationship between

the %VBD and the breast elasticity. The correlation thresholds that were used were in accordance with the recommended medical research thresholds (Mukaka 2012) and are presented in Table 4-6.

Table 4-6: Pearson's correlation coefficient correlation thresholds

Size of Correlation	Interpretation
.90 to 1.00 (-.90 to -1.00)	Very high positive (negative) correlation
.70 to .90 (-.70 to -.90)	High positive (negative) correlation
.50 to .70 (-.50 to -.70)	Moderate positive (negative) correlation
.30 to .50 (-.30 to -.50)	Low positive (negative) correlation
.00 to .30 (.00 to -.30)	Negligible correlation

Both the baseline %VBD and the breast elasticity data, according to the Shapiro-Wilks test, were normally distributed, and there were no outliers present in either dataset. Using Pearson's correlation coefficient, the results showed an *r*-value of 0.184 (p=0.589), this is a statistically insignificant negligible correlation between the two variables. The correlation is presented in Figure 4-7.

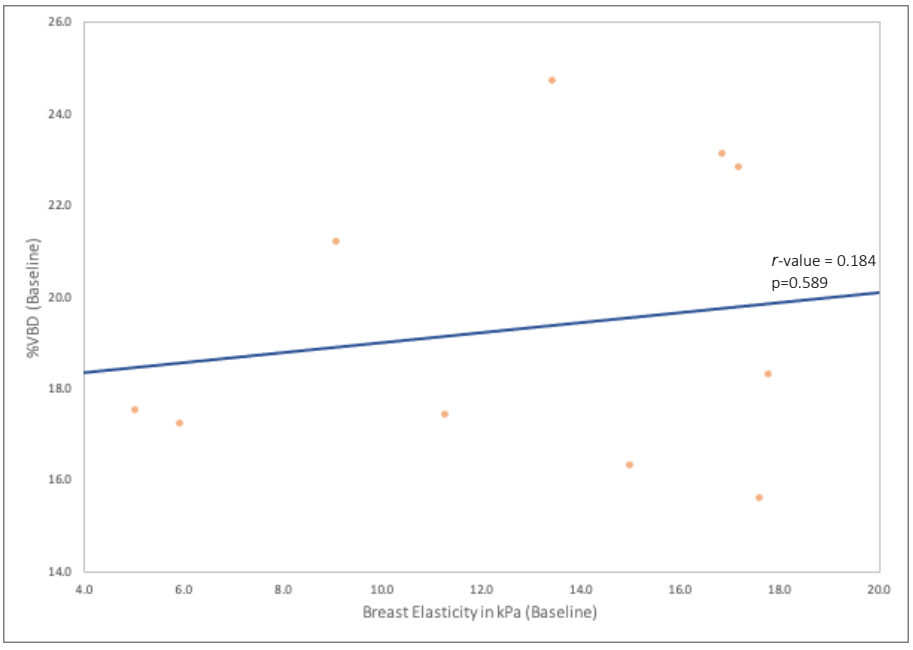

Figure 4-7: Correlation between %VBD and breast elasticity at baseline

Both the 12-month %VBD and three-month elasticity data, according to the Shapiro-Wilks test, was normally distributed. Using Pearson's correlation coefficient, the results showed an

r-value of 0.233 (p = 0.491), this is a statistically insignificant negligible correlation between the two variables. The correlation is presented in Figure 4-8.

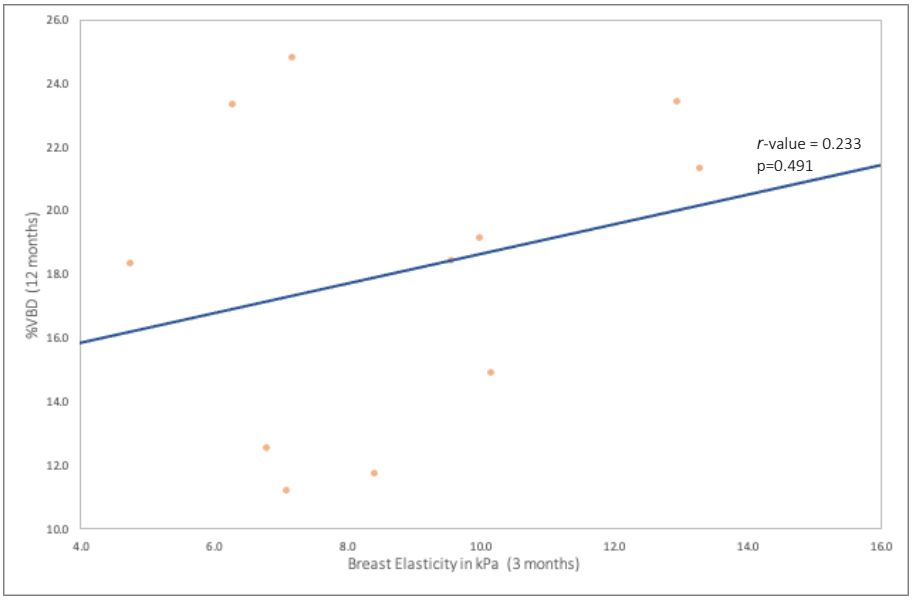

Figure 4-8: Correlation between %VBD at 12 months and breast elasticity at 3 months

When determining if there is a correlation between change in %VBD and the change in the elasticity values, the correlation analysis was conducted on the difference values between the baseline %VBD and 12-month %VBD results, and the difference values between the baseline elasticity and the 3-month elasticity values. The Shapiro-Wilk test for normality showed that the data were normally distributed; there were no outliers present within the data. Pearson's correlation for the change in the variables had an *r*-value of 0.315 (p=0.345); this was a statistically insignificant low positive correlation between the change in the variables across the study. The correlation is presented in Figure 4-9.

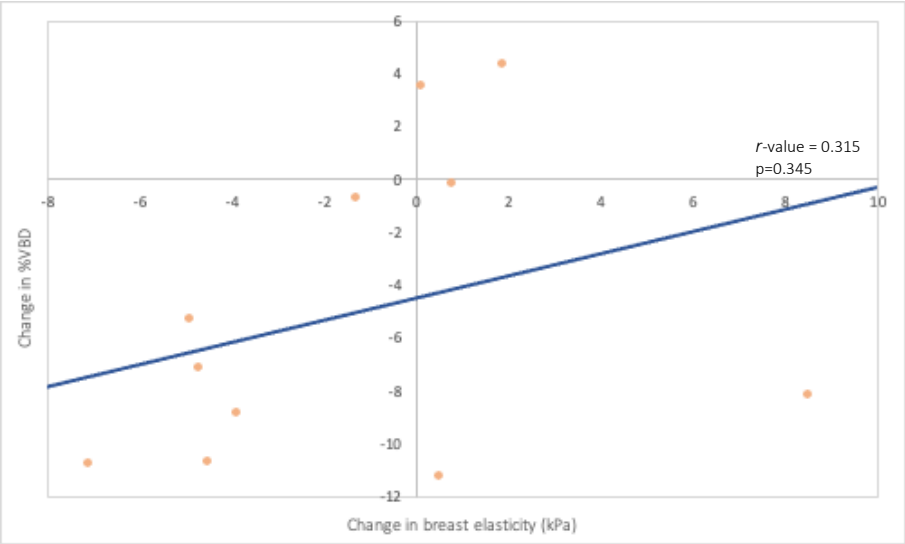

Figure 4-9: Correlation between change in %VBD (baseline to month 12) and change in breast elasticity (baseline to month 3)

4.4.6 Correlations Between the Total Fibroglandular Volume and Breast Elasticity

The TFV is the recorded volume of dense tissue within the mammogram, this is a variable of interest as the %VBD is influenced by both the fibroglandular volume and the TBV, so if a therapeutic intervention reduces both variables, the %VBD may remain unchanged. The TFV is the outcome measure that can quantify the extent of the changes occurring in the actual dense tissue itself; this being the tissue of interest in regard to interventions being used to reduce MBD.

Within the baseline TFV and the baseline breast elasticity values, the Shapiro-Wilks test showed that the baseline fibroglandular data were not normally distributed. There was a single outlier present within the data, and upon further analysis, this was deemed a correct figure and therefore was left in the analysis. Spearman's rank-order correlation was used as the statistical model. The results produced a r_s value of -0.118 (p=0.729); this was a statistically insignificant negligible correlation between the baseline fibroglandular volume values and the baseline breast elasticity values. The correlation is presented in Figure 4-10.

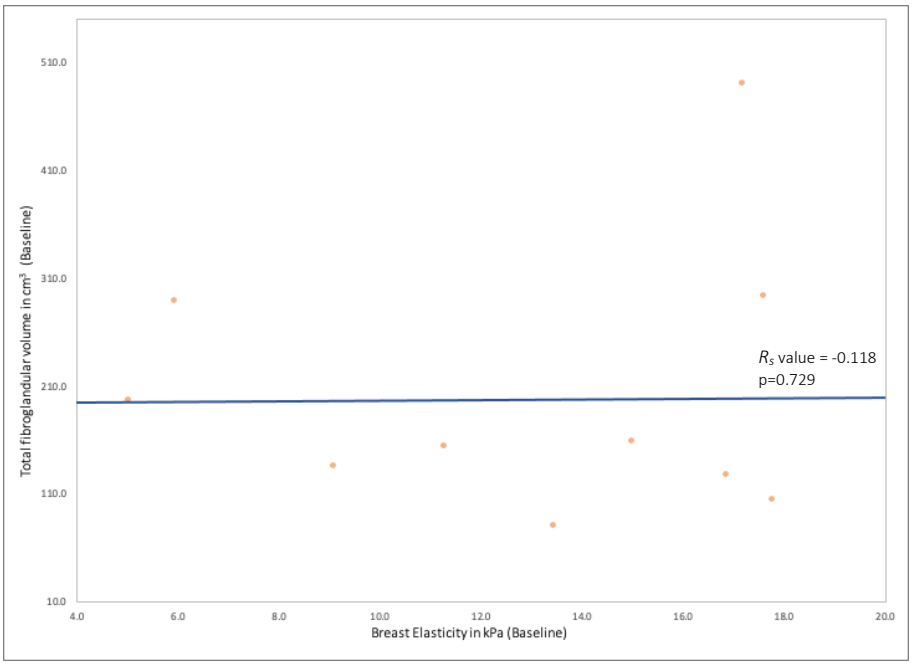

Figure 4-10: Correlation between total fibroglandular volume and breast elasticity at baseline

About the 12-month fibroglandular volume and 3-month elasticity data, the fibroglandular data, according to the Shapiro-Wilks test, was not normally distributed. No outliers were present within either dataset. Spearman rank-order correlation was used as the statistical model for the analysis. The results showed a r_s value of -0.436 (p=0.180) this was a statistically insignificant moderate negative correlation between the variables. The correlation is presented in Figure 4-11.

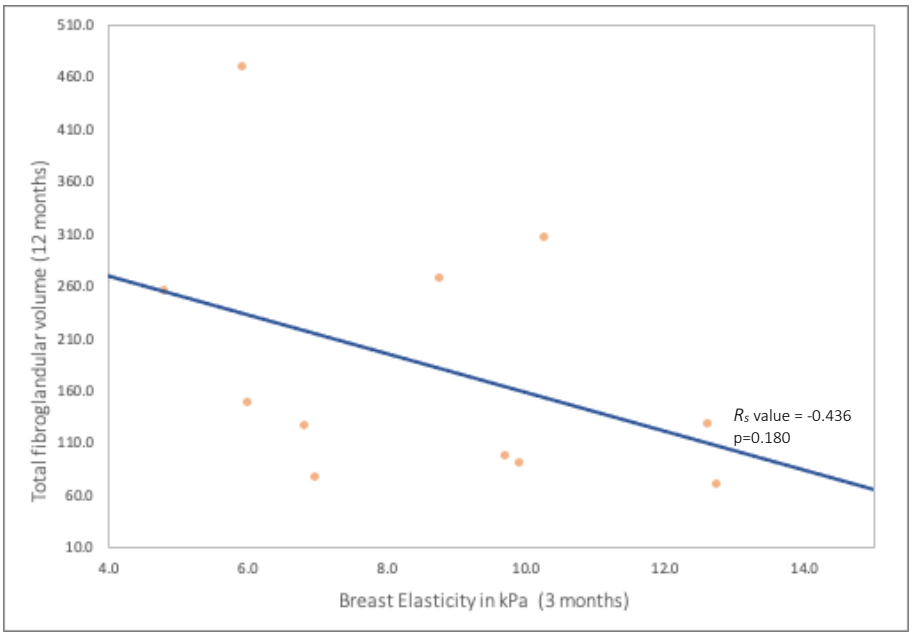

Figure 4-11: Correlation between total fibroglandular volume (month 12) and breast elasticity (month 3)

When determining if there was a correlation between change in TFV and the change in the elasticity values, the correlation analysis was conducted on the difference values of the baseline TFV and the 12-month TFV and the difference values of the baseline elasticity and the 3-month elasticity values. The Shapiro-Wilks test showed that the data were normally distributed. There was an outlier within the fibroglandular volume dataset, and with further analysis, this was deemed a true value and left in the analysis. Pearson correlation coefficient demonstrated that the *r*-value was 0.689 (p=0.019); this was a statistically significant moderate positive correlation between the two variables. The correlation is presented in Figure 4-12.

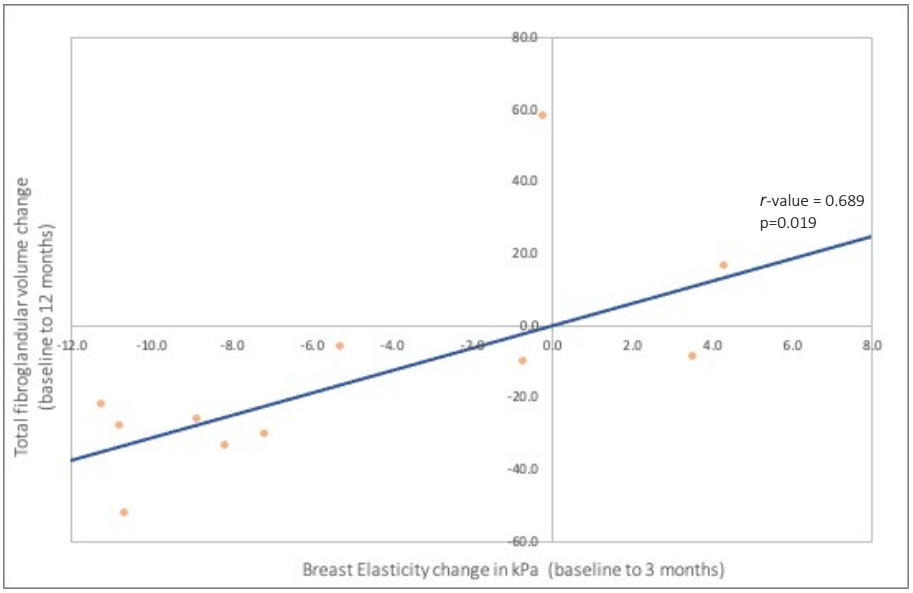

Figure 4-12: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 3)

In addition to the elasticity changes across the three months, two additional analyses were conducted for the one-month data and the two-month data elasticity change with the TFV changes across the 12 months. With the change from baseline to the one-month elasticity variables, the data were normally distributed according to the Shapiro-Wilk test, and there were multiple outliers within both datasets, which were deemed to be correct values and left in the analysis. Pearson's correlation coefficient produced an *r*-value of 0.611 (p=0.046). This was a statistically significant, moderate positive correlation between the two variables. The correlation is presented in Figure 4-13.

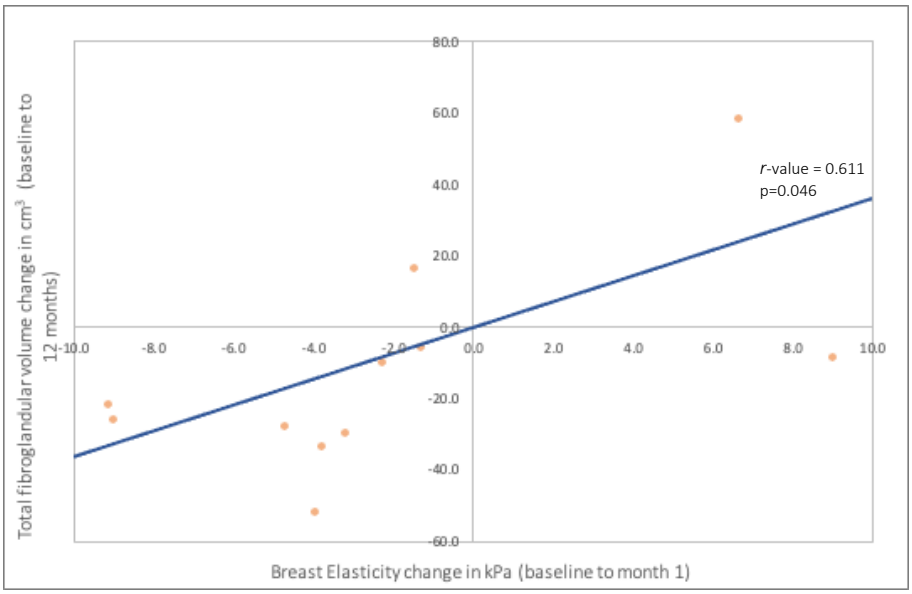

Figure 4-13: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 1)

The last analysis was the elasticity changes from baseline to month two, and the TFV changes from baseline to month 12, the data were normally distributed according to the Shapiro-Wilks test. There was a single outlier in the TFV dataset, which was assessed and deemed a true value and left in the analysis. Pearson's correlation coefficient produced an *r*-value of 0.818 (p=0.002). This shows that there was a statistically significant high positive correlation between the elasticity change two months and the TFV changes at 12-months. The correlation is presented in Figure 4-14.

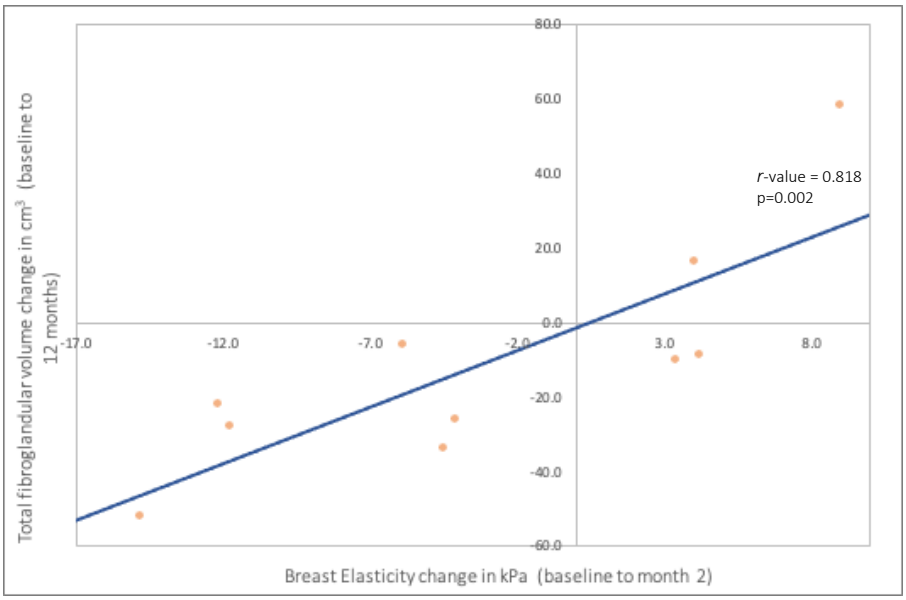

Figure 4-14: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 2)

4.5 Discussion

This was a sub-study within a single dose, single-centre, open-label, non-randomised pharmacokinetic trial of HAVAH*T+Ai*™ conducted on 11 premenopausal women. This study aimed to determine the pharmacodynamic effect on breast tissue elasticity, which is an experimental biomarker to determine breast tissue changes.

The breast tissue elasticity reduced progressively over time, with a relative reduction of 37% from baseline to the 3-month measurement. It has previously been reported that high MBD tissue shares similar characteristics to malignant breast tissue, specifically the fibrodense areas having a downregulation of fibroblasts, leading to a greater extracellular matrix component and low adipocytes (DeFilippis, Chang et al. 2012) and a decreased regulation of MMPs and their inhibitors TIMPs (Bonnans, Chou et al. 2014). This abnormal extracellular matrix deposition can lead to tissue stiffening, causing increased tissue elasticity as seen in breast cancer (Bonnans, Chou et al. 2014). We hypothesised that the characteristics of high MBD, such as high extracellular matrix content would lead to greater tissue elasticity; therefore, changes in elasticity may precede a change in MBD. These study results demonstrated that the HAVAH*T+Ai*™ combination had a rapid physiological effect on high breast tissue elasticity in this cohort of women with high MBD.

With the additional analyses that could be conducted with the 12-month follow-up mammogram, within this sample, the combination of HAVAH*T+Ai*™ descriptively produced an overall mean decrease in %VBD across the 12-months. In addition, the analyses showed that the TFV decreased over the 12 months, and this reduction was approaching the conventional benchmark for statistical significance with consistently large effect sizes across the participants. This decrease in TFV is of particular interest as the fibroglandular tissue is what constitutes the dense tissue, so a reduction in this tissue specifically may be able to reduce the breast cancer risk. Additionally, if an intervention can reduce the breast volume and the TFV, the %VBD value may not show a reduction, which could lead to Type II errors when accepting or negating the null hypothesis. Therefore, specifically seeing a change in this variable may beneficial.

One of the key objectives of this thesis is to determine if breast tissue elasticity can be used as a biomarker for MBD. Within this study, we were able to conduct correlation analyses on the changes in the breast elasticity with the changes in %VBD and the changes TFV. From these analyses, it showed that there was a statistically significant moderate correlation with the change in elasticity over three months to the change in TFV over the 12 months. The results also showed a moderate and strong correlation with the elasticity changes at month one and month two, respectively, with the TFV changes at 12 months. These result shows great promise that the elasticity changes may be able to represent and predict the density changes in a timelier manner, which is one of the strongest reasons to research breast elasticity as a biomarker for breast tissue changes. However, this study was unable to inform us as to what would happen to the elasticity values across a full 12 months. Furthermore, as this study was not sufficiently powered, we are unable to statistically demonstrate the significance of the results. Future research needs to be conducted with a formally powered sample size to demonstrate whether this relationship exists.

One of the limitations of this study, as previously mentioned, is the sample size, as this was a pilot study, no formal sample size calculation was completed. This unmeasured statistical power means that although the results can show a sign of effect, the results are unable to show the level of significance and need to be interpreted with caution. A further limitation of this study is the lack of previous research for the use of SWE for this indication. When using SWE, there are a variety of *post-hoc* analysis techniques that can be used to calculate breast elasticity. However, SWE hasn't been used to measure and evaluate changes for this indication, so no validated techniques have been published in the literature. Some of the results that were produced during this trial appeared to have a substantial amount of variation in the within-subject repeat measures. As this machine has not been used previously and the design of this trial was Phase 1, with no control group, we are unsure if this variability is natural fluctuations, measurement error, or the true results of the intervention on the breast tissue elasticity. In order to distinguish what may be causing the fluctuations, a reliability study, in women not receiving any hormonal intervention that could affect the breast, controlling for fluctuations in the participant's menstrual cycle needs to be completed. This additional information will allow us to calculate the intra-rater reliability and determine if there are natural fluctuations within the breast tissue elasticity.

As mentioned, another limitation with the SWE machine is the lack of validated protocols to produce the whole breast elasticity results. When using the SWE machine, once the image has been saved, Q-box™ are placed on the image, these produce the mean, max, min, SD elasticity of the area within the Q-Box™. These are customisable regarding the diameter and the location they are placed, and there is also the option of tracing the image to develop a customised shaped Q-Box™. There is no set protocol to utilise the Q-Box™ to find the whole breast average elasticity. By changing the number, diameter and location of the Q-Box™, the elasticity values can change. Future studies need to be conducted to analyse the best method to find the average breast elasticity. This further information could aid future studies, to find a reliable and consistent method to determine the elasticity, which will be especially beneficial when designing longitudinal trials with repeat elasticity measures to detect true change.

4.6 Conclusion

Overall, this pilot study generated evidence to show an association between a reduction in breast elasticity and a singular dose of HAVAH*T+Ai*™. There is also early evidence that these changes may correlate with the changes occurring in TGV in response to the same intervention.

4.7 Future Studies

Future studies need to be conducted to firstly determine consistency in the response of elasticity with another intervention that can affect MBD variables. In addition, due to the fluctuations seen in this study, a reliability study looking at the behaviour of breast elasticity, not on any interventions that can influence the breast tissue, needs to be conducted to determine if these fluctuations are, as mentioned, measurement error or typical findings. Studies of this nature can be found in Chapter 6 and Chapter 8 of this thesis.

Chapter 5 Anastrozole and GTx-024: The Effect of an Aromatase Inhibitor and Selective Androgen Receptor Modulator on Mammographic Breast Density and Breast Elasticity in Premenopausal Women

5.1 Background

In Chapter 4, in response to the therapeutic intervention of T and anastrozole (HAVAH*T+Ai*™), it was demonstrated that mean breast elasticity and mean TFV decreased within the recruited cohort of women. Further to this, the decreases in the mean breast elasticity were seen as early as the one-month repeat measurement. This early data is promising for the primary objective of this thesis and has generated initial findings and hypotheses, that can be further developed. As with all new, novel ideas, further research is required to validate the present findings, decrease the impact of research biases on the results and generate new evidence to contribute to the overall body of evidence. To determine if breast elasticity can be influenced by therapeutic interventions targeted at reducing MBD and determine if these changes correlated with the changes in MBD and the VolparaDensity™ variables, it needs to be established whether breast elasticity can be modified with a different intervention than HAVAH*T+Ai*™. By establishing whether a different intervention produces similar results with breast elasticity and MBD changes, we can show consistency with the response, which will contribute to demonstrating that breast elasticity may be a valid biomarker for MBD.

In addition to validating the findings, this chapter aimed to work through some of the limitations discussed in Section 4.5, one of which was the timing of the outcome measurements; the main issue being the omission of a 12-month SWE ultrasound to measure breast elasticity. As the schedule of assessments in Chapter 4 varied between the SWE breast elasticity measurements and the mammography variables, a study that had both variables measured at the same time points needed to be conducted to accurately calculate the relationship between the variables, and also to analyse the behaviour of breast elasticity across a 12-month time period.

This chapter is the second experimental trial conducted for this research program. A different hormonal combination was used to reduce oestrogenic drive and shift the E/A ratio towards an androgenic environment. This hormonal combination utilised an Ai (anastrozole) to block the conversion of androstenedione to E_1 and testosterone to E_2. The second component of the combination was a SARM (GTx-024 also called enobosarm). As discussed in Chapter 1, the rationale for using a SARM is that Ai's are contraindicated in premenopausal women due to perturbations in the homeostatic mechanisms controlling ovarian function (Casper 2007). Thus, by administering GTx-024, we postulated that the impact of an Ai on the hypothalamic-pituitary axis would be circumvented and not result in ovarian hyper-stimulation, allowing the combination to reduce MBD without undue side effects.

5.2 Objectives

The primary objective of this trial was to determine whether the combination of enobosarm and anastrozole reduces the VolparaDensity™ MBD measurements of %VBD and TFV and breast elasticity in kPa as measured by SWE.
The secondary objective of this trial was to determine whether the changes (if changes are observed) in the VolparaDensity™ mammography variables (%VBD and TFV) correlate with the changes in the breast elasticity in kPa as measured by SWE.

5.3 Research Questions

5.3.1 Primary Research Questions

1. Does the combination of GTx-024 and anastrozole reduce %VBD and TFV in premenopausal women with high MBD?
2. Does the combination of GTx-024 and anastrozole reduce breast elasticity in kPa in premenopausal women with high MBD?

5.3.2 Secondary Research Questions

1. Do the changes in %VBD and TFV correlate with the changes in breast elasticity?

5.4 Method

5.4.1 Research Design and Ethical Approval

This trial was designed to obtain data regarding the effect of anastrozole and GTx-024 in a patient population in a clinical situation, during a 12-month dosing regimen. Given the lack of previous studies researching this treatment regimen, a single-centre, open-label pilot study with a small group of premenopausal women was deemed the most suitable design to determine any signal of effect. Bellberry Limited HREC approved the study (approval number 2016-02-099), and all patients provided informed consent. The study has also been registered on Clinicaltrials.gov (clinical trial identifier: NCT032646651).

5.4.2 Patient Population

The trial population consisted of premenopausal women. Participants were referred to the clinic for either the evaluation of high MBD or breast pain or both factors. Participants were screened for eligibility for study participation during the 21 days prior to the scheduled dosing date. At the screening visit, medical histories (including menstrual history) and demographic data, including sex, age, race, body weight (kg), and height (cm) were recorded. Other assessments included physical examination, breast examination, mammography (if not performed within the previous three months, utilising VolparaDensity™ analysis), complete vital signs, and laboratory tests as specified. Participants were eligible if they had premenopausal levels of FSH/LH/E_2, VolparaDensity™ volumetric breast density of ≥ 15.5% (combined average both breasts) and breast pain in the previous month of equal to or greater than 40mm on a 100mm VAS for pain.

5.4.3 Treatment Regimens

Patients were scheduled to be treated daily for a 12-month period with the investigational product of both GTx-024 (GTx Pharmaceuticals, Memphis, TN) 9mg a day as three x 3mg soft gel capsules and anastrozole 1mg a day as singular a solid tablet. These treatments were dispensed monthly, and treatment compliance was recorded for each participant. The

investigational product was labelled according to the Australian Code of Good Manufacturing Practice for medicinal products. As the study was open label, no blinding was required.

5.4.4 Outcome Measures

As per the methodology in Chapter 4, MBD was measured using VolapraDensity™, which analyses a digital mammogram to provide the measurements of TFV in cm^3, TBV in cm^3 and from these measurements %VBD is calculated. Figure 5-1 is the visual output that is produced with VolparaDensity™. Mammograms were performed at baseline and month 12. Shear wave elastography was conducted using SuperSonic™ Imagine Aixplorer®® ShearWave™ ultrasound machine (Aixplorer®, France). The methodology for the SWE was described in Section 4.3.3. Shear wave elastography was done at baseline, month one, month three and month 12. Breast pain was measured using a 100mm VAS for breast pain (Appendix 2). Menopausal symptoms were measured using the Menopause Rating Scale (MRS) (Appendix 3) at baseline, month one, month three and month 12.

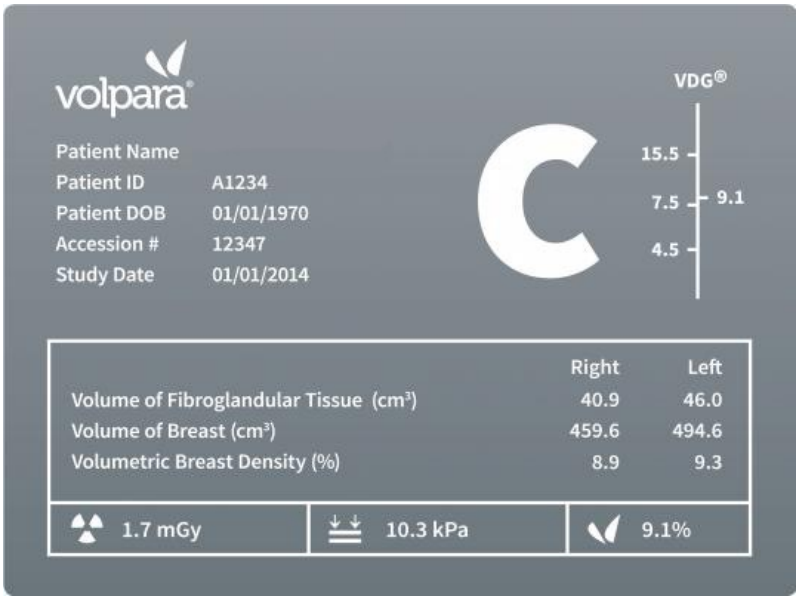

Figure 5-1: VolparaDensity™ Output

Blood tests were conducted at baseline, month one, month three, month six, month nine and month 12 for haematology, biochemistry and a hormonal profile including LH, FSH, E2, progesterone, total testosterone, FAI, SHBG. Blood tests were done by Clinpath Laboratories at Burnside Hospital and analysed at the corresponding laboratory. Serum plasma anastrozole levels were measured at months nine and 12. This was done by CPR Pharma Services Pty Ltd

(now known as Agilex Biolabs Pty Ltd), South Australia. All AEs were documented. The Schedule of assessments and procedures are presented in Table 5-1.

Table 5-1: Schedule of Assessments and Procedures

Trial Month[a]	Physical exam[b] Medical History Serum Pregnancy Test[c]	Mammography[d]	Urine Pregnancy	Haem[e] And Bio	Hormonal Profile[f]	Shear Wave Ultrasound[g]	Breast Pain/Stiffness[h]	Menopause Questionnaire[i]	Vital Signs[j]	AE[k] and Con-Meds
Screening	X	X		X	X				X	
Baseline (pre-dose)			X			X	X	X	X	X
1 Months				X	X	X	X			X
3 Months			X	X	X	X	X	X		X
6 Months				X	X					X
9 Months				X	X					X
12 Months (end of study)		X	X	X	X	X	X	X		X
15 Months (Follow-up)			X	X	X		X	X		X
End of trial/Early termination										

a Assessments were to be made in the following order: Vital signs, adverse events and blood sampling.

b Physical examination includes breasts, height, weight and BMI calculation.

c Serum pregnancy test.

d Unless mammography performed in previous three months using VolparaDensity™ analysis on a New Generation Hologic mammogram machine (Dr Jones & Partners at Burnside War Memorial Hospital or St Andrews Hospital).

e Safety laboratory tests: haematology and biochemistry.

f Hormonal profile included LH, FSH, E_2, progesterone, FAI, dehydroepiandrosterone sulphate (DHEAS) and SHBG. Repeat measurement of progesterone was to be carried out, 3 days post result, in participants not in the luteal phase of their menstrual cycle at the time of first assessment.

g Assessment of breast tissue elasticity. The pre-dose assessment denoted the baseline.

h Breast pain/stiffness as measured by a 100mm visual analogue pain scale.

i As measured by menopause rating scale

j Vital signs included seated systolic and diastolic blood pressure, pulse rate and body temperature.

k Adverse Events and Concomitant Medication were collected, following consent, at every visit and unsolicited for the duration of the trial.

5.4.5 Data synthesis and Analytical Procedures

All data were tabulated into Microsoft Excel 2016 (Microsoft, USA). SPSS version 25 (IBM, USA) was used to compute the descriptive statistics and inferential statistics. The significance level was set *a priori* with a p-value equal or less than 0.05, with a 95% CI presented for the primary and secondary analyses. All subjects were included in the analysis using the intention-to-treat principle. Population sample characteristics were reported using descriptive statistics.

The distribution of the data was assessed via the Shapiro-Wilk test with a p-value greater than 0.05 determining the data were normally distributed. Outliers were determined by a visual assessment of a box plot; any determined outliers were further analysed to be grouped as true values or measurement errors; if the values were deemed true values, they were left within the analysis.

The pre- and post-intervention data for the mammography variables (%VBD, TFV, TBV) were analysed using a paired samples T-test for parametric data; the Wilcoxon-Signed Rank test was used for non-parametric data. The breast elasticity at each of the four time points was analysed using a one-way repeat measure analysis of variance (ANOVA) with *post-hoc* Bonferroni adjustments for parametric data. Friedman test was the alternative statistical model for the one-way repeat measure ANOVA, for non-parametric data. Correlation analyses were conducted using Pearson's correlation coefficient for the parametric data. Spearman's Rank Order Correlation Coefficient was used for non-parametric data.

5.5 Results

5.5.1 Participant Characteristics

Eight participants were recruited during the period of January 2017 to May 2017. Table 5-2 presents the participant demographic characteristics. The average treatment compliance was 97.7% (SD 3.7%), with 12.5%of participants having 100% compliance. One participant withdrew from the study during the tenth month for reasons not related to the investigational product.

Table 5-2: Participant Characteristics

Demographic Parameter (Units)	Average (SD)
Age (years)	40 (3.2)
Weight (kg)	64.6 (11.1)
Height (m)	168.4 (2.5)
BMI (kg/m²)	22.8 (3.5)
%VBD	21.70% (3.7)
Pain (VAS)	71.13 (16.4)
MRS	12.86 (6.2)
% of Participants	
Gender (Female)	100%
Race (White)	100%
Race (White/Asian)	0%
Ethnicity (Not Hispanic or Latino)	0%

%VBD = Percentage volumetric breast density as measured with VolparaDensity™
VAS = Visual analogue scale
MRS = Menopause rating scale

5.5.2 Mammography Variables

5.5.2.1 Percentage Volumetric Breast Density

Table 5-3 presents the mammography variable data. The %VBD data were normally distributed, and no outliers were present in the data. The participants had a greater baseline %VBD of 21.7% (SD 3.7%) compared to the end of study (EOS) values of 18.5% (SD 4.7%), this was a decrease of 3.2% (95% CI -6.67 to 0.26; p = 0.065). This result had a Cohen's D effect size of 0.78.

Table 5-3: Mammography Variable Values

	Mean value (SD)	Mean Change from Baseline (SD)	95% CI	p-value
%VBD				
Baseline	21.7% (3.7)			
12 Month	18.5% (4.8)	-3.2% (4.1%)	-6.67% to 0.26	0.065
Total Fibroglandular Volume				
Baseline	226.3cm^3 (112.1)			
12 Month	131.6cm^3 (82.0)	-94.7cm^3 (33.7%)	67.01 to 122.45	<0.005
Total Breast Volume				
Baseline	1115.4cm^3 (235.9)			
12 Month	794.8cm^3 (660.6)	-320.6cm^3 (131.8%)	-430.84 to -210.42	<0.005

%VBD = Percentage volumetric breast density as measured with VolparaDensity™

5.5.2.2 Total Fibroglandular Volume

Table 5-3 presents the TFV variable data. The TFV data was positively skewed, with a Shapiro-Wilk test result showing a p-value of 0.034; thus, the data was not normally distributed. There were no outliers present within the data. The Wilcoxon-Sign Rank test was used to analyse the data. The median TFV was greater at baseline with a value of 185.1cm^3 compared to the 12-month time point with a median value of 111.4cm^3. There were eight negative differences, and zero positive differences and ties. This was a median difference of -79.4cm^3 with a p-value of 0.008.

5.5.2.3 Total Breast Volume

Table 5-3 presents the TBV data. The TBV data were normally distributed as per the Shapiro-Wilk test with a p-value of 0.735. There were no outliers present within the data. Total breast volume was greater at baseline with a value of 1115.4cm3 (SD 667.6cm^3) compared to the 12-month (end-of-study) values of 794.8cm^3 (SD 660.3cm^3). This was a change of -320.6cm^3 (95% CI -430.8cm^3 to -210.4cm^3; p<0.005). This result had a Cohens D value of 0.65.

5.5.3 Average Whole Breast Elasticity

Table 5-4 presents the average and median whole breast elasticity values at each time point. The baseline, month three and month 12 data were not normally distributed; the month one data were normally distributed.

Table 5-4: Whole breast elasticity average, median and test for normality values

Time Point	Average Elasticity in kPa (SD)	Median Elasticity in kPa	Shapiro-Wilk Test p-value
Baseline	17.9 (9.4)	17.2	0.015
Month 1	14.3 (4.8)	13.5	0.396
Month 3	13.9 (7.8)	12.8	0.000
Month 12	12.6 (5.3)	11.9	0.048

Within the four datasets, there were five outliers that were greater than one-point-five box lengths from the edge of the boxplot; these were in the baseline, month three and month 12 dataset. These were deemed to be correct values and were not excluded from the analysis. There was an extreme outlier in the three-month dataset; this is classified as being greater than three box lengths from the edge of the box. Again, this was deemed to be a correct figure and was left in the analysis. Due to the data being non-parametric, the Friedman test was used to analyse the data.

The results from the Friedman test showed that breast elasticity was statistically significantly different at the different time points in response to the GTx-024 and anastrozole hormonal combination with a p-value of 0.003. Pairwise comparisons were performed with Bonferroni corrections for multiple comparisons. There were statistically significant differences between the baseline and the 12-month elasticity data with a p-value of 0.001. The complete results from the pairwise comparisons are presented in Table 5-5.

Table 5-5: Results of the whole breast elasticity Friedman pairwise comparisons

Pairwise Comparison	p-value
Baseline – 1 month	0.490
Baseline – 3 months	0.147
Baseline – 12 months	0.001

5.5.4 Correlations Between Mammography Variables and Breast Elasticity

Correlation analyses were run between the mammography variables and the whole breast elasticity data. All of the correlation coefficient results are presented in Table 5-6. The most notable correlation was between the change in breast elasticity from baseline to month 12 and the change in TFV from baseline to month 12; this resulted in a moderate correlation with a *r*-value of 0.586, this correlation had a p-value of 0.127 (Figure 5-2). In this analysis, the data of the change in TFV was not normally distributed and was transformed using logarithmic transformation. The correlation analysis between the change in breast elasticity from baseline to one month and the change in TFV from baseline to month 12 resulted in an *r*-value of 0.500, with a p-value of 0.313. Lastly, the change in breast elasticity from baseline to month one and the change in TBV from baseline to month 12 with a *r*-value of 0.585, with a p-value of 0.223.

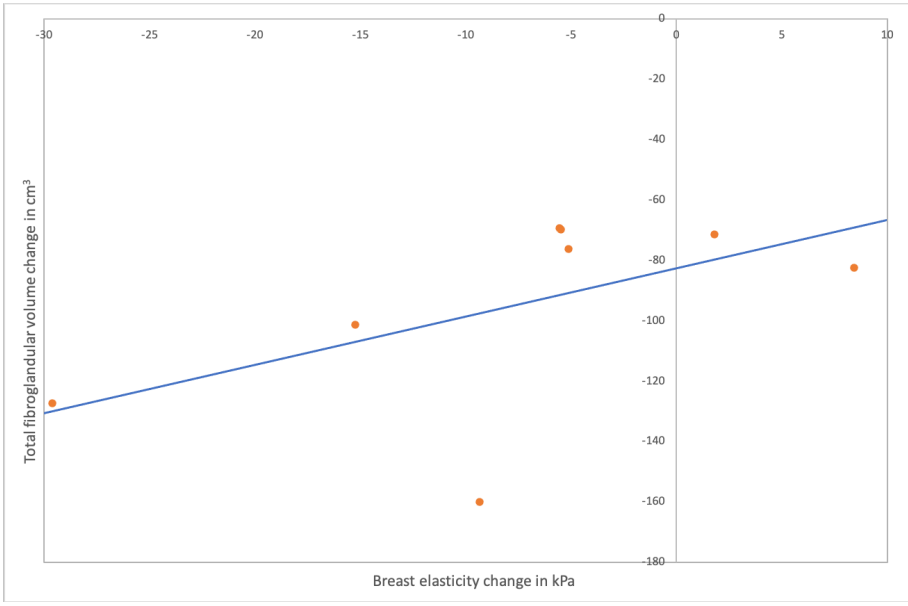

Figure 5-2: Correlation between total fibroglandular volume change (baseline to month 12) and elasticity change (baseline to month 12)

Table 5-6: Correlation coefficient results between breast elasticity and mammography variables

Correlation	Pearson's R	p-value
Baseline Correlations		
Breast elasticity and TBV	0.246	0.557
Breast elasticity and TFV	0.470	0.221
Breast elasticity and %VBD	0.264	0.528
End of Study Correlations		
Breast elasticity and TBV	0.854	0.007
Breast elasticity and TFV	0.842	0.009
Breast elasticity and %VBD	0.273	0.523
Baseline to End of Study Change Correlations		
Breast elasticity and TFV	0.586	0.127
Breast elasticity and TBV	0.280	0.502
Breast elasticity and %VBD	-0.12	0.997
Elasticity Change at 1 month and all other Baseline to End of Study Change Correlations		
Breast elasticity and TFV	0.500	0.313
Breast elasticity and TBV	0.585	0.223
Breast elasticity and %VBD	-0.451	0.262

%VBD – Percentage volumetric density as analysed with VolparaDensity™

5.5.5 Blood Analysis

All blood serum hormone results are presented in Appendix 4; LH, FSH, E_2, progesterone, testosterone and DHEAS remained stable through the study, with no out of range values. Free androgen index was increased at all time points except for baseline; the mean values at month six, nine, and 12 being 7.44%, 10%, 6.53%, respectively. Sex hormone binding globulin was significantly decreased at all time points in 87.50-100% of the participants, the mean values at month one, three, six, nine and 12 being 12.88nmol/L, 11.63nmol/L, 10nmol/L, 11.00nmol/L, 11.63nmol/L, respectively. Serum plasma anastrozole levels were 30.09ng/ml (SD 9.42ng/ml) at nine months and 29.74ng/ml (SD 7.59ng/ml) at 12 months.

5.5.6 Adverse Events

There was a total of 88 AEs across all eight participants. There were 11 AEs that were deemed related or possibly related to GTx-024 and anastrozole, and these are all listed in Table 5-7. These AEs ranged from mild to moderate severity, the most commonly occurring AEs being increased alanine aminotransferase (ALT) (47u/L, 125 u/L and 98 u/L). There were no serious adverse events, deaths or withdrawals from the study due to any AEs.

Table 5-7: List of adverse events (AEs) related

Commonly Occurring Drug Related Adverse Events	**Number of Events**
Mild Severity	
Night Sweats	2
Acne	2
Voice changes	2
Hair Loss	1
Moderate Severity	
Increased ALT	3
Voice changes	1

5.6 Discussion

This is the first study utilising GTx-024 and anastrozole therapy in premenopausal women for the reduction of MBD. This initial evidence for the proof of concept regarding the efficacy of this combination was demonstrated in both the primary endpoint of %VBD and TFV and the secondary endpoint of breast elasticity. In regards to the primary research question, the results showed that %VBD had an average reduction of -3.2%, this is a relative reduction of -14.75% which is above the 10% MBD reduction required for breast cancer risk reduction benefits as stated by Cuzick, Warwick et al. (2011). This reduction was also trending towards the conventional level of statistical significance with a p-value of 0.065. Following the decreases in %VBD, the TFV had a statistically significant reduction of -79.35cm^3, which was a relative reduction of 41.86%. This TFV reduction is more than double the 20% reduction in the dense area seen in women treated with tamoxifen, which lead to a 50% reduction in breast cancer mortality (Li, Humphreys et al. 2013). Furthermore, TBV had a statistically significant

reduction of -320.63cm^3, this being an absolute reduction of 28.75%. These are significantly large decreases that have been seen in a small number of women and indicate that this treatment may be favourable for the indication of reducing MBD variables. The significant decrease in TFV is of great relevance as this reduction represents a decrease in the stromal and fibroglandular part of the breast tissue; this being the tissue of interest regarding MBD and the reductions that lead to beneficial effects in breast cancer risk. These results indicate that the combination of GTx-024 and anastrozole is able to reduce %MBD, TBV and TFV in a small population of women. Having validated the effect of this combination on MBD variables, the next stage is to analyse how the breast elasticity responds to the same hormonal intervention and how these changes correlate with the changes in the mammography variables.

The second objective of this study was determining whether the hormonal combination of GTx-024 and anastrozole, in addition to the MBD variables, was able to reduce breast elasticity. For this research objective, there were also favourable results, as the findings show that there was a statistically significant reduction in breast elasticity at the 12-month measurement, with a reduction of -5.37kpa, this being a relative reduction of 30.4%. There were also decreases in breast elasticity reported at each time point with a reduction of -3.69kPa (relative reduction of 20.6%) at one month and -4kPa (relative reduction of 22.3%) at the three-month time point. This investigational product is the second hormonal combination that has decreased both MBD variables and breast elasticity, which indicates that there is consistency in the response, which can further provide evidence that breast elasticity may be a valid biomarker for MBD. These results also further demonstrate that breast elasticity responds at a very early stage to the intervention. Within the first month, 67% of the reduction that occurred over the 12 months had already been observed. This early elasticity reduction may be used to show signs of early response. With this early response being favourable for the indication of determining patient response to interventions that reduce MBD. Aiding the decision that a woman may be a responder or a non-responder to the therapy and whether they should continue the therapy, as many of these interventions have undesirable side effect profiles.

The third part of this study was analysing whether the changes in %VBD and TFV correlate with the changes in breast elasticity. This analysis needed to be conducted to demonstrate

that the change in elasticity relates to the changes in the mammography variables. If there is the correlation, and as elasticity is responding to the intervention at such an early time point, breast elasticity may be able to predict the changes in %VBD and TFV. The results from this study show that there was an insignificant negative, weak correlation between the changes in breast elasticity with the change in %VBD. It was also analysed whether the changes in breast elasticity at one month correlated with the changes in %VBD across the 12-month intervention period. This analysis was done to determine if breast elasticity has the potential to be used as an early indicator for MBD changes. These results demonstrate that within this cohort, there is no correlation between these two variables.

However, when analysing the correlation between breast elasticity and TFV, there were more favourable results. The results showed that there was a moderate correlation at one month and at 12 months. Both of these findings were statistically insignificant, but as the study was underpowered, there is some data to promote further research that change in breast elasticity may correlation with changes in TFV, which is still beneficial for breast cancer risk and mortality reduction (Li, Humphreys et al. 2013).

These correlations with both %VBD and TFV, in particular, TFV showed that there is somewhat of a relationship. However, the aim of this research program is to validate elasticity to be used as an early indicator of response to interventions to reduce MBD as MBD takes an extended time to show significant changes. Longer studies may lead to a stronger correlation between these three variables, as there is more time for changes in %VBD and TFV to occur. Another explanation for the weaker than expected correlation between elasticity and %VBD is that %VBD is calculated with the TFV and TBV, which in this study both decreased. This indicates that the %VBD may not have changed at the same rate as the elasticity and the TFV variables.

As mentioned, some of these findings were statistically insignificant, however, this was a pilot, proof of concept pharmacokinetic study, so when observing the trends for the decreases and the correlations that were described, the results are favourable to generate and guide future studies. The findings within this study indicate that as the fibrodense region of the breast decreases, the breast elasticity may also decrease. This is favourable as with further research, elasticity may be used as a non-invasive and comfortable biomarker to determine the early

response of breast tissue to hormonal interventions. Thus, allowing healthcare providers to modify the treatment approach based on whether the patient is a responder or a non-responder. This knowledge can influence patient management, their health-related outcomes and the cost of the treatment (Manton, Chaturvedi et al. 2006).

This study does have some limitations which need to be taken into consideration when interpreting the findings. Firstly, as it was an early proof of concept pharmacokinetic study, only eight women were recruited. For this reason, this study was not powered to prove statistical significance; this meaning that although the results can show a sign of effect in regard to the breast tissue elasticity and the mammography variables, we cannot provide a definitive conclusion regarding the outcomes. A further limitation was the study design; it was an open-label, phase II drug trial, which did not have a control group. By not having a control group (and not controlling all variables), we are unable to determine if there was a causal relationship between the hormonal intervention and the effect on the breast tissue. For the same reason, we are also unable to report what would have occurred in the breast tissue of women not on the hormonal intervention. More rigorous research designs, such as an RCT, with a placebo-controlled group are required to determine if GTx-024 and anastrozole is the causal factor for the reduction in MBD and breast elasticity, and the subsequent reduction in breast cancer risk.

A further limitation of this study was the within-subject variation of the breast elasticity data. There appeared to be a substantial level of observed fluctuations in the SWE repeat measurements (which was also reported in Chapter 4). As there was no control group and no normative data from previous research using SWE for this indication, we are unable to determine if the elasticity values were reflecting the response of the breast tissue to the intervention, measurement error or natural fluctuations. Further research should done on healthy subjects, controlling for hormonal fluctuations to determine the normative values, and the normal fluctuations in breast tissue elasticity that may occur in a premenopausal patient population. This data is integral for the interpretation of these results and any future SWE results generated.

5.7 Conclusion

This study aimed to determine if enobosarm and anastrozole, a novel hormonal combination of an Ai and a SARM, used in premenopausal women with high MBD, could reduce %VBD, TFV and breast elasticity. This study generated evidence that this combination had signs of being efficacious at reducing %VBD, TFV and breast elasticity. Although not reaching the conventional threshold for statistical significance, there was a strong correlation between the changes in breast elasticity and TFV. These results are encouraging for breast elasticity being a viable biomarker for MBD. To further validate these findings, future research needs to be conducted with a more rigorously designed, adequately powered, placebo-controlled, double-blind RCT to determine if there was a causal relationship between GTx-024 and anastrozole and the reductions in %VBD, TFV and breast elasticity. Additionally, as suggested, further research also needs to be done to determine the natural fluctuations that occur with the elasticity of the breast across the month due to hormonal fluctuations when a woman is not on any hormonal interventions.

Chapter 6 Does the Region of Interest Size Affect the Breast Elasticity and Coefficient of Variation of a Data Set Analysing the Average Breast Elasticity of Women on Hormonal Chemopreventative Therapy?

6.1 Introduction

Shear wave elastography operates by having the ultrasound beams generate acoustic radiation force impulses (ARFI), which provide the mechanical excitation through pushing beams that deform the underlying tissue of interest. Several of these pushing beams are transmitted at different depths, which result in the propagation of transient shear waves. The speed of these shear waves is then measured using a scanner with a very fast frame rate, allowing the shear waves to be followed in real-time. This is repeated for different lines; allowing a map of a ROI to be created from analysing the differences in arrival times and calculating the shear wave speeds. A colour-coded image is then displayed on the SWE monitor, a Q-Box™ which is a measuring tool is placed on this image, which provides an area for the elasticity variables to be calculated. The quantitative data is presented as a measure of shear wave speed in m/s^{-1} or converted to the Young's Modulus and displayed as kPa (Bercoff, Pernot et al. 2004, Bercoff, Tanter et al. 2004, Sebag, Vaillant-Lombard et al. 2010, Shiina, Nightingale et al. 2015).

To date, within the field of breast imaging, the predominant use of SWE is differentiating between benign and malignant lesions. For this indication, the common technique to quantify the tissue elasticity is to use a circular Q-Box™ that usually ranges in size from 2-3mm in diameter, which is then placed over the stiffest part of the lesion (it may also include the area immediately adjacent to this region). Another Q-Box™ is then placed on an adjacent area of fatty tissue to provide a reference value (Chang, Moon et al. 2011, Berg, Zhang et al. 2012, Youk, Gweon et al. 2013, Au, Ghai et al. 2014). This is a practical technique as it has been found that, with exceptions, malignant masses are of a greater elasticity than benign, so it is imperative that the quantitative values of the stiffest region of the lesion are known. A further use of SWE is determining the effectiveness of neoadjuvant chemotherapy on malignant breast lesions, for which the same technique as differentiating benign or malignant breast

lesions is used. However, this indication uses repeat measures to quantify the changes occurring in the elasticity values (Evans, Armstrong et al. 2013, Jing, Cheng et al. 2016, Ma, Zhang et al. 2017).

Few studies have used SWE to calculate the average breast elasticity of the entire breast and not just a particular lesion. In current literature, the techniques chosen to measure breast elasticity with the absence of a lesion, have been to use either the outer upper region of the breast (Li, Wang et al. 2015) or divide the breast into four quadrants (Rzymski, Skórzewska et al. 2011, Rzymski, Wysocki et al. 2011, Rzymski, Wilczak et al. 2012) and place one 3mm Q-Box™ on an area of the image that represents the glandular tissue and one that represents the fatty tissue, which provides the minimum, mean, maximum and SD of both the fatty and the glandular tissue and additionally the glandular to fatty ratio. There are weaknesses with using these techniques. Firstly, the different regions of the breast have varying amounts of glandular and fatty tissue and it has been seen that the mean elasticity of the glandular tissue can vary in the different quadrants (some areas having greater or lower elasticity). Secondly, the elasticity values are variable throughout each SWE image; relying on one Q-Box™, again, may not provide an accurate representation of the true mean elasticity for each quadrant of the breast and subsequently, the whole breast. Using this technique can also increase the operator's influence on the elasticity values, as the operator can bias the data by placing the Q-Box™ on an area of greater or lesser elasticity, whichever favours their intentions. This operator dependence is particularly problematic for longitudinal studies or studies analysing the efficacy of therapeutic interventions, as unless the Q-Box™ location is kept constant, unreliable findings can be reported.

6.2 Objective

Currently, there is no published literature reporting the differences in the mean elasticity values between differing breast quadrants and the impact of using different sized Q-Box™ or the number of Q-Box™ on the overall elasticity values. As there is increasing interest in researching whole breast elasticity for a variety of indications, there needs to be a standardised protocol in regard to the breast quadrants used and Q-Box™ size, frequency and location on the SWE image. This will improve the reliability and accuracy of calculating the mean whole breast elasticity. The objective of this research is to determine if using different

post-hoc analysis protocols can significantly change the overall elasticity values and to find a protocol which provides the most precise data attenuation for future research.

6.3 Method

6.3.1 Patient Demographics

The data was acquired from a collection of SWE images of 11 women who were participants of a clinical trial at Wellend Health Pty Ltd (Chapter 4 – HAVAH*T+Ai*™ study), South Australia (Bellberry Limited Human Research Ethics Committee approval number 2017-06-434). Participants of this trial all provided permission for their images and data to be used for subsequent research. Women were recruited into the initial trial if they had a VolparaDensity™ %VBD of ≥ 15.5% (combined average both breasts), aged between 33-55 years inclusive, body weight between 50 and 90kg inclusive and in good general health. Please refer to Chapter 4, Section 4.3.1 for full eligibility criteria.

6.3.2 Shear Wave Elastography Imaging Protocol

Breast SWE was conducted using the SuperSonic™ Imagine Aixplorer® ShearWave™ elastorgaphy machine (Aixlorer, France). Four images were taken per breast, one in each quadrant (Fig. 6-1), the transducer head was held still for 5 to 10 seconds in order for the shear waves to propagate through the tissues. A generous amount of contact gel was used to prevent artefactual stiffness from being recorded. Please refer to Chapter 4, Section 4.3.3 for further details regarding the method for the capturing of the SWE images.

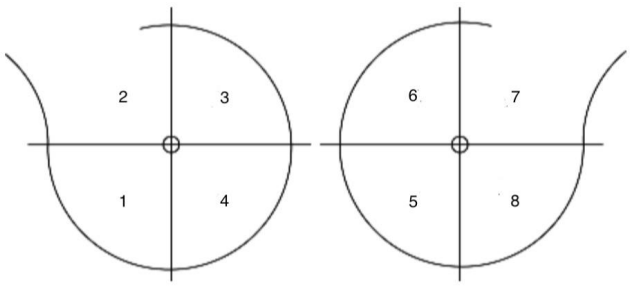

Figure 6-1: Image sequence of the breast quadrants used in shear wave elastography

6.3.3 Elasticity Data Generation

Seven different protocols were used to generate the elasticity data from the SWE images. The protocols used were a circular Q-Box™ with varying diameter and frequencies, the Q-Box™ trace function and the Q-Box™ ratio function. The original protocol that was used in Chapter 4 and 5 of this thesis was the 6 x 3mm Q-Box™ placed evenly across the image (Figure 6-2), this then determined the minimum, maximum, mean and standard deviation of the tissue elasticity measured in kPa and the depth of the placing of each Q-Box™. The 6 x 3mm Q-Box™ protocol, as stated in Section 4.3.3, was advised as the method to be used by the Medical Director of Wellend Health Pty Ltd.

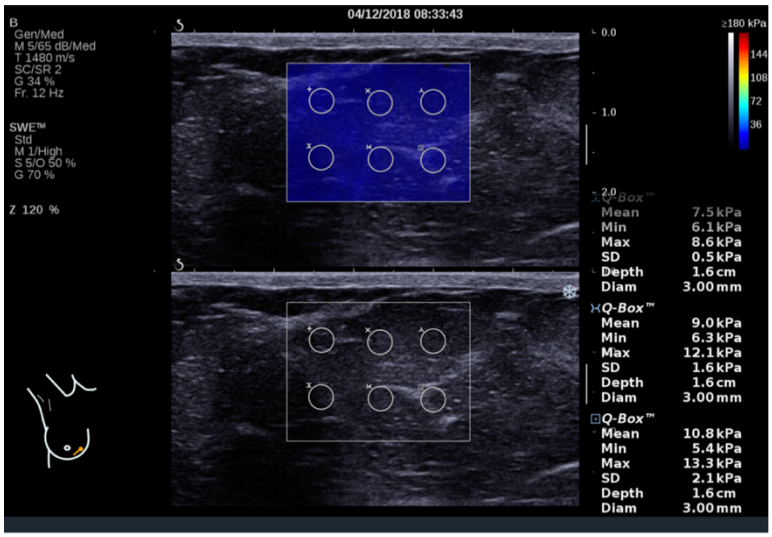

Figure 6-2: Shear Wave Elastography Image with 6 x 3mm Q-Box™ places evenly over the image

From the 6 x 3mm Q-Box™, the mean elasticity was calculated per quadrant, per breast and the overall breast elasticity. The same calculations were conducted using six, six-millimetre (6 x 6mm) Q-Box™ (Figure 6-3), one ten millimetre Q-Box™ (Figure 6-4), using the Q-Box™ trace function to trace the entire image (Figure 6-5), and using the Q-Box™ trace to trace the desired ROI, not including the elasticity artefacts and 'black holes', which are locations within the image where the elasticity does not correspond to the B-mode images and produces extremely high elasticity figures or where shear waves have not been calculated, leaving the elasticity as zero, respectively (Figure 6-6). These different protocols were chosen for a variety of reasons; the diameter of the other Q-Box™ were arbitrary in value but were used to determine if altering the size and frequency could have a greater clinical utility or a greater level of reliability. The Q-Box™ trace function was chosen, as on face value, appeared as

though it would be time-efficient and as it incorporates the largest area of the breast easily omitting any artefacts and black holes, it was hypothesised that it could generate the most valid data. The Q-Box™ trace function was also a technique used by Evans (2015) for research utilising SWE for whole breast elasticity, which was presented in a conference paper.

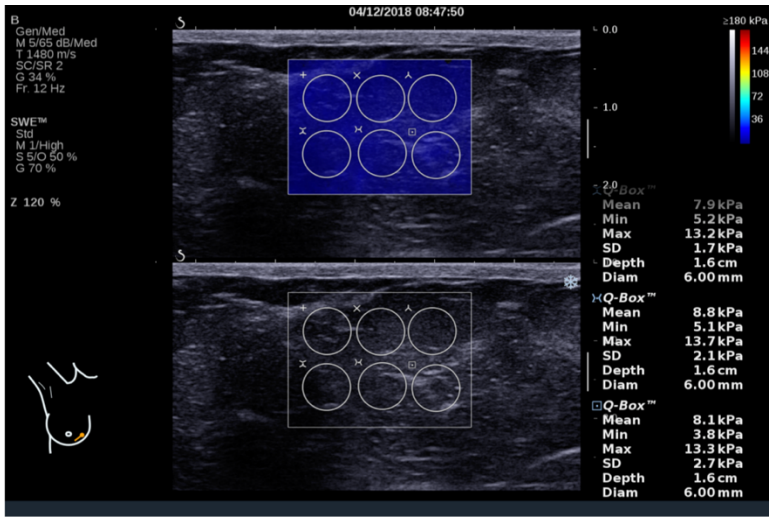

Figure 6-3: Shear Wave Elastography image with 6 x 6mm Q-Box™ es places evenly across the image

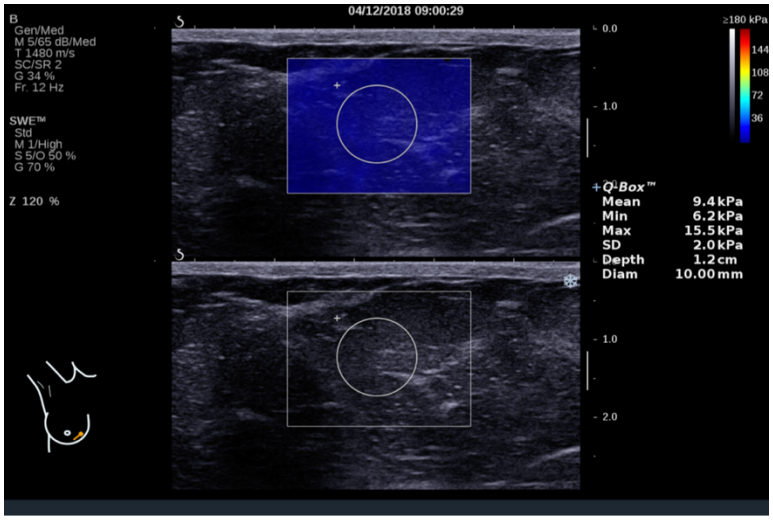

Figure 6-4: Shear Wave Elastography Image with one 10mm Q-Box™ placed in the middle of the image

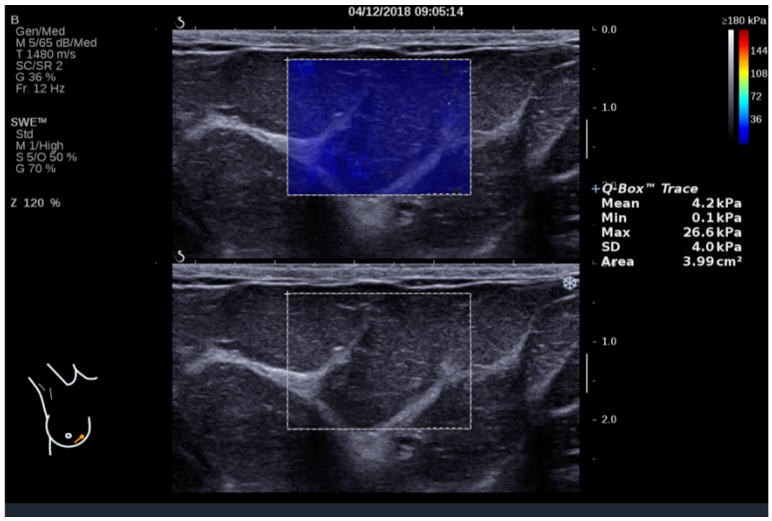

Figure 6-5: Shear Wave Elastography image with a Q-Box™ traced around the border of the region of interest

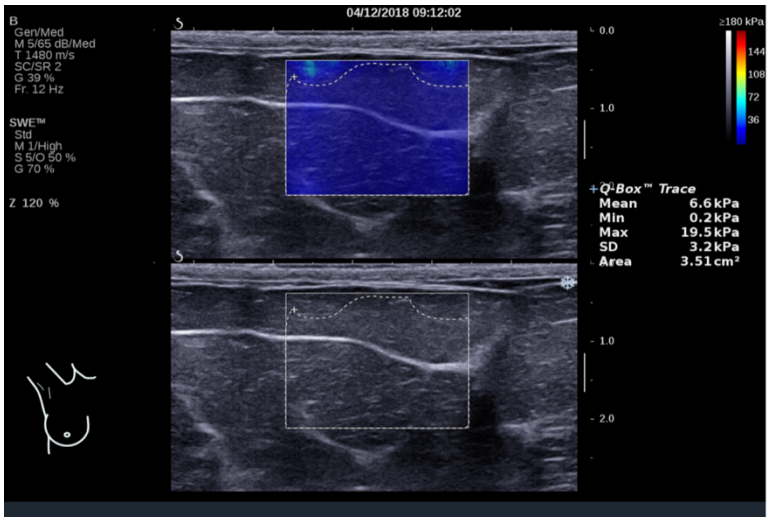

Figure 6-6: Shear Wave Elastography image with the Q-Box™ traced within the region of interest, avoiding artefacts or black holes

The last two methods of calculating the elasticity were using the Q-Box™ ratio function, firstly placing one 3mm Q-Box™ on an area that represents glandular tissue and another 3mm Q-Box™ on an area that represents predominately fatty tissue (Figure 6-7). Within the image, fatty tissue appeared black on the B-mode image and dark blue on the shear wave image. Glandular tissue appeared white on the B-mode image and light blue on the shear wave image. This technique provided the minimum, maximum, mean elasticity of both the glandular and the fatty tissue in addition to the glandular-to-fatty elasticity ratio. The Q-Box™ ratio function was then repeated, this protocol was slightly altered with a customised sized Q-Box™ (Figure 6-8); the size of the Q-Box™ depended on the area of the glandular and the fatty tissue. This protocol was done to ensure the Q-Box™ only contained the tissue of interest. For

this study, the elasticity from the singular glandular tissue Q-Box™ was used as the breast elasticity data; the actual ratio was not included in this analysis.

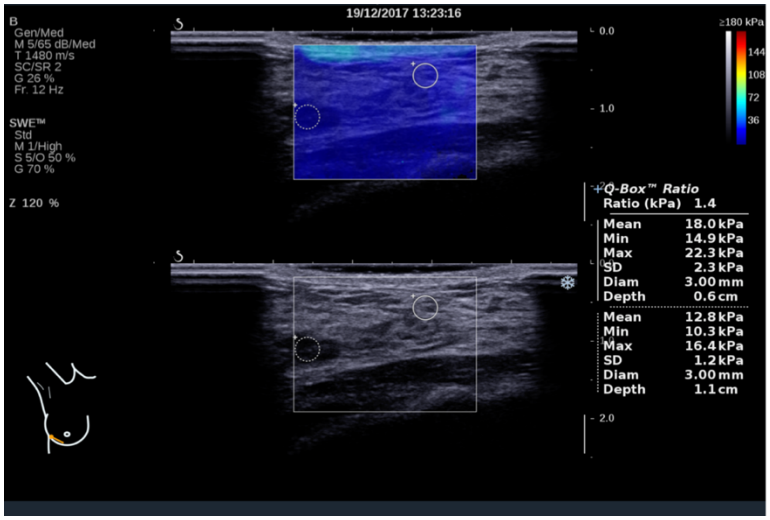

Figure 6-7: Shear Wave Elastography image using Q-Box™ ratio function, placing a 3mm Q-Box™ on an area of glandular tissue and an area of fatty tissue

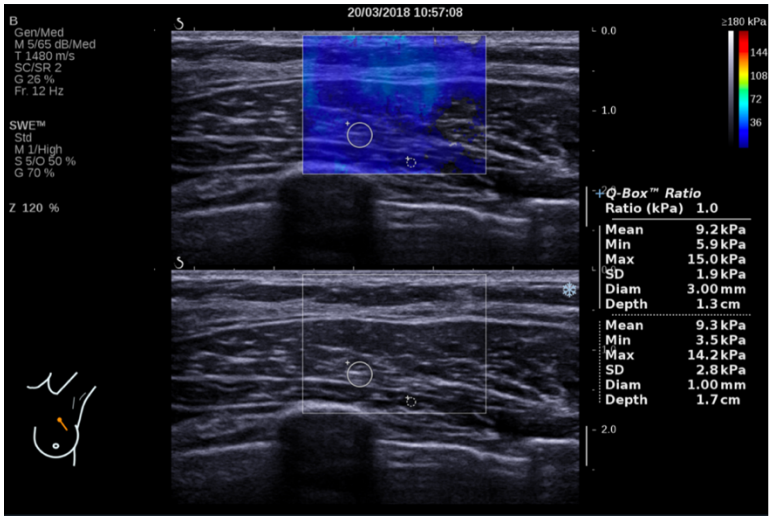

Figure 6-8: Shear Wave Elastography Image using Q-Box™ ratio function, placing a 3mm Q-Box™ on an area of glandular tissue and a 1mm Q-Box™ on an area of fatty tissue. This image also shows areas where the shear wave hasn't propagated

6.3.4 Statistical Analysis

The data were entered into Microsoft Excel (2016), and statistical analysis was conducted using SPSS 25 (IBM, New York, USA). The 6 x 3mm Q-Box™™ protocol was used as the reference standard for the analyses, while the other protocols are the index tests. The data were analysed using repeated measures ANOVAs with Bonferroni *post-hoc* analysis to determine if there was a statistically significant difference between the reference standard

and the index test, this analysis was done for all of the different quadrants of the breast, left and right breast and both breasts combined. In addition, the coefficient of variation (CV) was calculated for each protocol, and this provided the levels of dispersions of each of the variables. A Bland-Altman plot was used to find the level of agreement between the references standard and the other variables. Where applicable, statistical significance was determined with a p-value ≤ 0.05, and a confidence interval that did not cross zero.

6.4 Results

6.4.1 Participant Demographics

The data for this chapter were collected from the 11 participants that were recruited for the study in Chapter 4. All the participant demographic data have previously been reported in Section 4.4.1. All participants had their data included in all the analyses.

6.4.2 Determining the Reference Standard

As reported in Section 6.2, the objective of this study was to determine the best method of extracting the breast elasticity data from the shear wave elastography image. The 6 x 3mm Q-Box™ was decided as the reference standard as it was the method previously advised as the method to use to collect elasticity data from the Medical Director at Wellend Health Pty Ltd, who worked closely with the Supersonic Aixplorer® Adelaide, South Australia representative.

The initial analysis was looking at the whole breast combined dataset; this was the mean of the left and right breasts. The descriptive statistics are presented in Table 6-1.

Table 6-1: Descriptive statistics for average breast elasticity for each protocol

Variable	Mean (SD) in kPa
6 x 3mm Q-Box™	10.90 (4.35)
6 x 6mm Q-Box™	11.30 (4.30)
1 x 10mm Q-Box™	9.71 (3.780)
ROI Full trace	12.21 (4.81)
ROI Free-Trace	10.68 (3.60)
Ratio Glandular Q-Box™	13.51 (4.71)
Alternative Ratio Glandular Q-Box™	11.30 (3.70)

The whole breast elasticity dataset was normally distributed, and there were no outliers present within the data. According to Mauchly's test for sphericity, the sphericity of the data was violated. The Epsilon (ε) was 0.605, as calculated according to Greenhouse and Geiser (1959) and was used to correct the one-way repeated measure ANOVA. The breast elasticity values from a number of the difference protocols were statistically significantly different from the 6 x 3mm Q-Box™™ protocol, F (3.318, 142.661) = 40.833, p<0.0005. In regard to the pairwise comparison, all the results can be found in Table 6-2.

Table 6-2: Pairwise comparison of all protocol elasticity data

Q-Box™ Reference Protocol	Index Protocol	Mean Difference (kPa)	95% Confidence Interval	p-value
6 x 3mm Q-Box™	6 x 6mm Q-Box™	-0.40	-0.82 to 0.02	0.081
	1 x 10mm Q-Box™	1.19	0.68 to 1.70	<0.0005
	ROI Full Trace	-1.31	-2.12 to -0.50	<0.0005
	ROI Free-Trace	0.22	-0.30 to 0.74	1.000
	Ratio	-2.61	-3.40 to -1.82	<0.0005
	Alternative Ratio	-0.40	-1.45 to 0.66	1.000

When comparing the 6 x 3mm Q-Box™ data to the 6 x 6mm Q-Box™ data, there was a statistically insignificant mean difference of -0.40kPa (95% CI -0.82 to 0.02; p = 0.081). This demonstrates that although the 6 x 6mm data provides a slightly lower mean elasticity value, there were no real differences between the protocols. The comparison between the 6 x 3mm Q-Box™ data to the 10mm data revealed a statistically significant mean difference of 1.19kPa

(95% CI 0.68 to 1.70; p<0.0005). This result demonstrated that the 10mm Q-Box™ consistently gave higher readings than the 6 x 3mm protocol. There was a statistically significant mean difference between the 6 x 3mm Q-Box™ data and the full-box trace data with a mean difference of -1.31kPa (95% CI -2.12 to -0.50; p<0.0005). This result demonstrates that the full-box trace produced consistently lower whole breast elasticity values when compared to the 6 x 3mm Q-Box™ data. There was a statistically insignificant difference between the 6 x 3mm Q-Box™ and the Free-Trace Q-Box™ data of 0.22kPa (95% CI -0.29 to 0.74; p=1.000), as with the 6 x 6mm Q-Box™ data, there was no real difference between the Free-Trace Q-Box™ data and the 6 x 3mm Q-Box™ data. There was a statistically significant difference of -2.61kPa (95% Ci -3.40 to -1.82; p<0.0005) between the original ratio data and the 6 x 3mm Q-Box™ data. Interestingly, there was a statistically insignificant difference of -0.40kPa (95% CI -1.45 to 0.66; p=1.000) between the alternative ratio data and the 6 x 3mm Q-Box™ data.

As there seemed to be a significant difference between the original ratio data and the alternate ratio data, the results from the repeat measures ANOVA were recorded for these two variables. When comparing the original Q-Box™ ratio data to the alternative Q-Box™ ratio data, there was a statistically significant mean difference of 2.22kPa (95%CI 1.08 to 3.35; p<0.0005). This result alluded to the hypothesised inconsistency that can occur when using the Q-Box™ ratio as a technique to generate the breast elasticity data. This is due to this technique using a singular Q-Box™, and the fluctuating elasticity within a particular ROI; so, the positioning of the Q-Box™ can significantly influence the elasticity data. This may be why there is a statistically significant difference between the data using the original ratio and the alternate ratio protocol.

The next stage of the analysis was to determine the coefficient of variation for the data generated using each of the protocols. The coefficient of variation is a measure of the dispersion of the data and is defined as the ratio of the SD to the mean. The protocol which generates the lowest CV shows a greater level of precision than the remaining protocols. The CV results are presented in Table 6-3. The protocol with the lowest coefficient of variation was the whole breast Free-Trace Q-Box™.

Table 6-3: Coefficient of variations of elasticity protocols

Elasticity Protocol	Mean Coefficient of Variation % (SD)
6 x 3mm Q-Box™	51.80% (8.81)
6 x 6mm Q-Box™	52.22% (19.80)
1 x 10mm Q-Box™	39.76% (11.94)
Q-Box™ Full Trace	31.02% (9.77)
Free-Trace	30.96% (9.22)
Original Ratio Data	39.27% (10.33)
Alternative Ratio Data	34.28% (9.17)

6.4.3 Bland Altman Plots

The next stage of this analysis is using a Bland Altman plot. A Bland Altman plot was proposed in 1983 (Altman and Bland 1983) and is used to describe the agreement and precision of two quantitative measurements by creating limits of agreement. These limits of agreement are calculated using the mean and the standard deviation of the differences between the two measurements. The Bland Altman plot is interpreted informally; however, Altman and Bland (1983) recommended that 95% of the data points should lie between the limits of agreement. When interpreting the plot, the solid line represents the bias, which is computed as the breast elasticity value generated by one of the protocols minus the value of the other method. Ideally, this bias should be close to zero; however, if not, it indicates that the two methods are systematically producing different results. In addition, when interpreting the plot, the limits of agreement and the bias need to be considered in a clinical manner, if the bias is large and the limits of agreement are wide, this may indicate the results are ambiguous, and the SWE protocol is not a valid method to generate the breast tissue elasticity. The 6 x 3mm Q-Box™ protocol remains the reference standard for the Bland Altman plots. Figure 6-9 shows the Bland Altman plot of the 6 x 6 mm Q-Box™ protocol and the 3 x 3mm Q-Box™ Protocol.

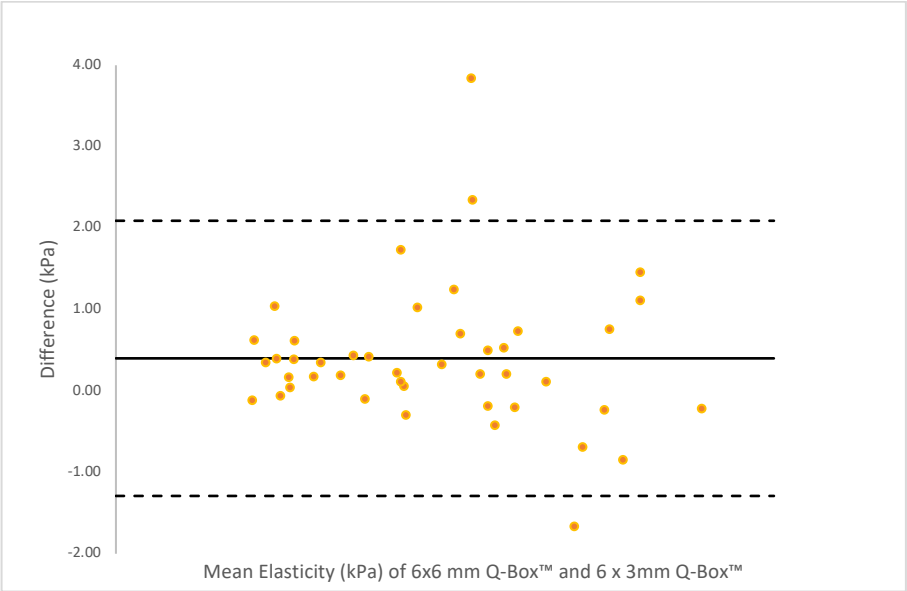

Figure 6-9: Bland Altman plot with 6 x 6mm Q-Box™ and 6 x 3mm Q-Box™ protocol data

For this plot, the bias is 0.4kPa which is a relatively small bias and would not impact the clinical interpretation of the data. The upper limit of agreement was 2.09kPa, and the lower limit of agreement was -1.29kPa, there is a large spread of the data points above and below the bias, and there are three data points that fall outside the 95% limits of agreement. There also is appears to be a larger spread of the data as the elasticity increases, which may represent a proportional bias. Figure 6-10 shows the Bland Altman plot of the 10mm Q-Box™ data and the 6 x 3mm Q-Box™ protocol data.

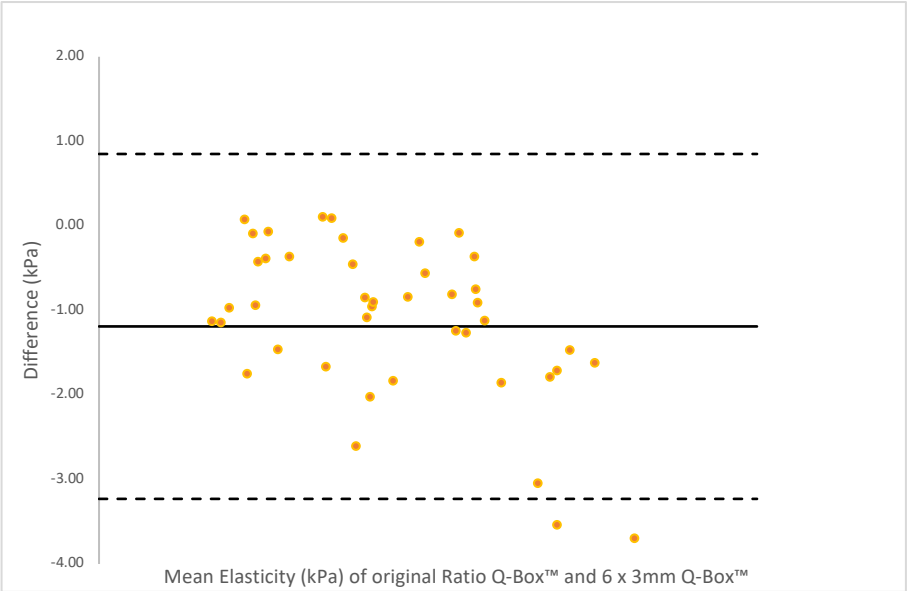

Figure 6-10: Bland Altman Plot with 10mm Q-Box™ and 6 x 3mm Q-Box™ protocol data

This plot has a larger bias of -1.19kPa, which demonstrates that the measures are producing systematically different elasticity measurements. The limits of agreement are also clinically relevant as the lower limit is -3.23kPa, which shows the measurement error with the 10mm Q-Box™ would produce ambiguous results as the limits are large enough to make the interpretation of the results by clinicians or researchers difficult; as they would not be able to determine if changes in elasticity are relating to a true change in the tissue or due to measurement error. Figure 6-11 shows the Bland-Altman plot of the full-box trace Q-Box™ data and the 6 x 3mm Q-Box™ protocol data.

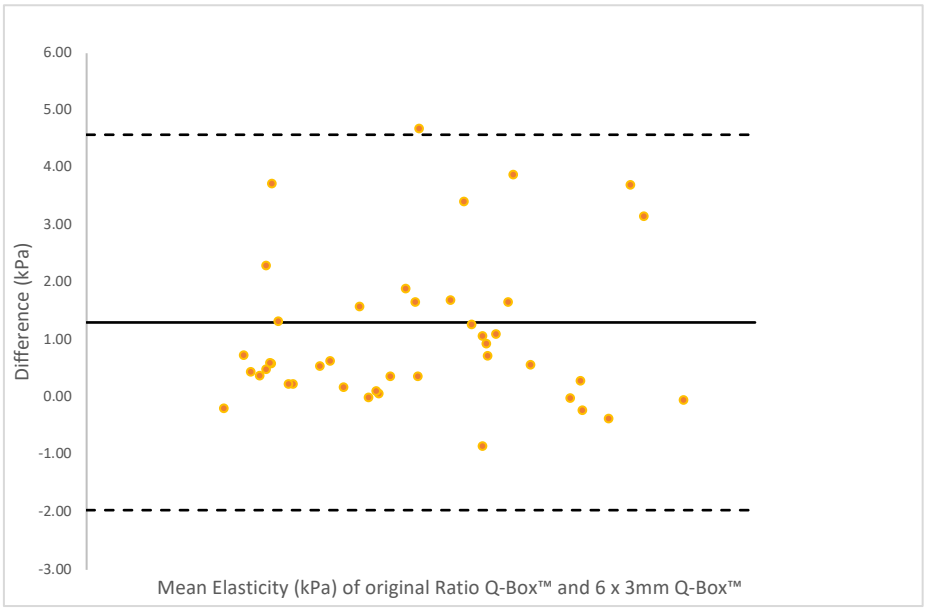

Figure 6-11: Bland Altman plot with Full-Box Trace Q-Box™ and 6 x 3mm Q-Box™ protocol data

The Bland-Altman plot for the full-box trace Q-Box™ shows that the bias is quite high with a value of 1.31kPa and the upper limit of agreement is 4.58kPa. Again, this shows that using this method to generate elasticity data would not be appropriate for clinical trials as the limits of agreement are so wide any changes observed would be ambiguous as we would not be certain the changes recorded are due to changes in the tissue or just measurement error. There is also substantial spread within the data, which shows a lack of precision within the data collected. There is also a proportional bias as there appears to be a larger spread of the data as the elasticity increases. Figure 6-12 shows the Bland Altman plot of the Free-Trace Q-Box™ data and the 6 x 3mm Q-Box™ protocol data.

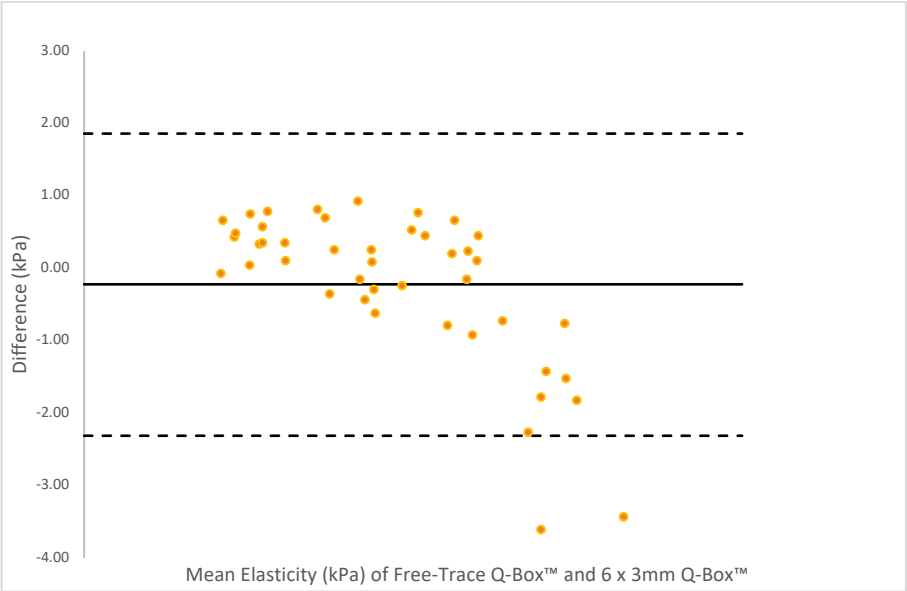

Figure 6-12: Bland Altman plot with Free-Trace Q-Box™ and 6 x 3mm Q-Box™ protocol data

The Bland-Altman plot of the Free-Trace Q-Box™ data and the 6 x 3mm Q-Box™ data show a small bias with a value of -0.22kPa, which demonstrates that the two methods of generating elasticity data are essentially equivalent. The limits of agreement are also lower than some of the other variables. This plot shows that as the elasticity increases the differences also increases, which could indicate a proportional bias is present within the data. Upon doing a linear regression, there was a *r*-value of 0.585 with a p-value of <0.0005, showing that statistically no proportional bias was present. Figure 6-13 shows the Bland Altman plot of the original ratio Q-Box™ data (glandular tissue) and the 6 x 3mm Q-Box™ protocol data.

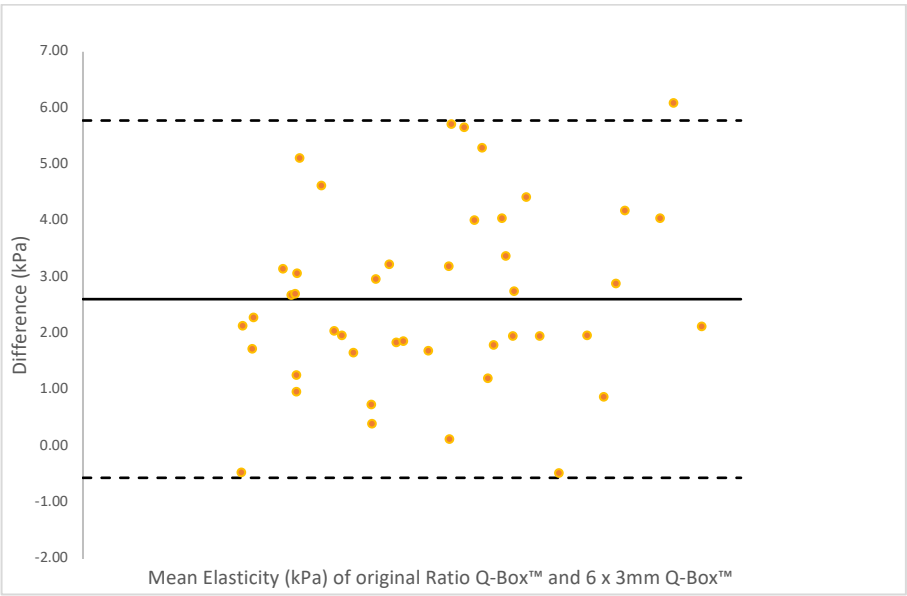

Figure 6-13: Bland Altman plot with Original ratio glandular Q-Box™ and 6 x 3mm Q-Box™ protocol data

The Bland-Altman plot of the original ratio Q-Box™ data (glandular tissue) and the 6 x 3mm Q-Box™ protocol data shows that the data lacks precisions with the data points scattered around the bias and through the limits of agreement. The bias itself is large with a value of 2.61kPa, and the limits of agreement are very wide showing that any results produced using this method are ambiguous and may or may not reflect changes within the tissue of measurement error. Figure 6-14 shows the Bland Altman plot of the alternative ratio glandular Q-Box™ data and the 6 x 3mm Q-Box™ protocol data.

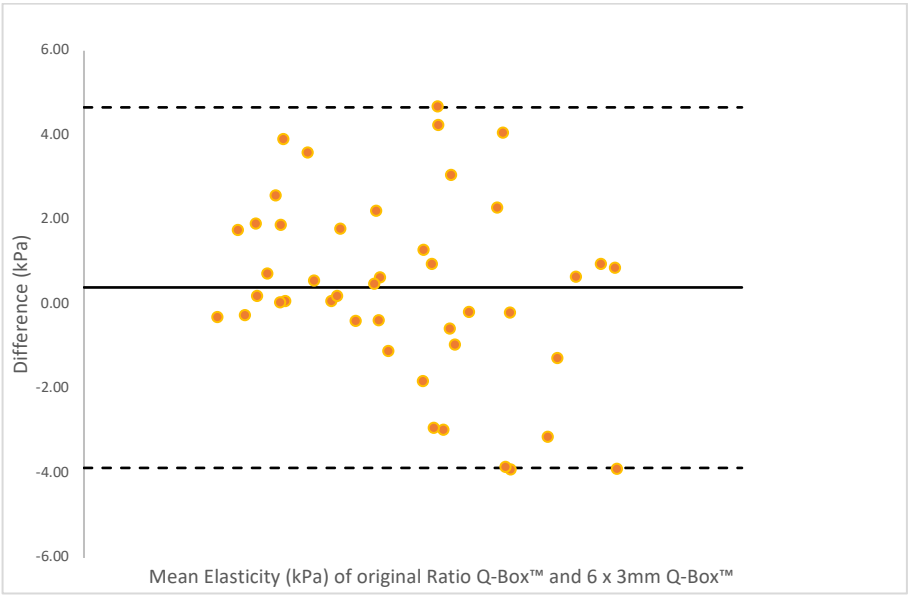

Figure 6-14: Bland Altman plot with Alternative ratio glandular Q-Box™ and 6 x 3mm Q-Box™ protocol data

The Bland-Altman plot of the alternative ratio Q-Box™ data (glandular tissue) and the 6 x 3mm Q-Box™ protocol data shows that the limits of agreement are wide and as reported with other methods, this demonstrates that results gathered using this method of breast elasticity generation are ambiguous as we are unable to determine if the change has come from changes in the tissue elasticity or just due to measurement error.

Out of all the Bland Altman plots presented and the visual, objective interpretation that followed, it was decided that the greatest level of agreement is between the Free-Trace Q-Box™ protocol and the reference standard of the 6 x 3mm Q-Box™ protocol. The 6 x 6mm Q-Box™, due to the level of agreement, may also be a valid measurement protocol.

6.4.4 Comparison of Quadrants

The next analysis conducted was to determine if there was a difference in the value of each quadrant, as some studies use a singular quadrant to determine the breast elasticity. This analysis was conducted for the 6x3mm Q-Box™, 6x6mm Q-Box™ and the Free-Trace Q-Box™.

6.4.4.1 6 x 3mm Q-Box™

The initial analysis was to determine if the data were normally distributed. According to the Shapiro-Wilks analysis, the data were not normally distributed. Results from the Shapiro-Wilks test are presented in Table 6-4.

Table 6-4: 6 x 3mm Q-Box™ Shapiro-Wilks test for normality values

Quadrant	Shapiro-Wilk p-value
Lower Outer	0.045
Upper Outer	0.061
Upper Inner	0.000
Lower Inner	0.090

As the data was not normally distributed a non-parametric statistical model was chosen. The Friedman Test was used to determine if there were differences between the breast quadrants. The descriptive statistics are presented in table 6-5.

Table 6-5: 6 x 3mm Q-Box™ descriptive statistics for comparison of values between breast quadrants

Breast Quadrant	Median Elasticity (kPa)
Lower Outer	10.03
Upper Outer	11.40
Upper Inner	9.78
Lower Inner	10.06

According to the Freidman Test, the breast elasticity was statistically significantly different within the different breast quadrants $X^2(3) = 8.12$, $p=0.043$. To conduct the pairwise comparisons, the Wilcoxon Signed-Rank Test was used, so the number of measurements were not taken into account. In order to use the Wilcoxon Signed-Rank Test to determine if there is a statistically significant median difference between two related groups, the shape of the distribution of the median differences should be symmetrical. All of the differences in the

following results were symmetrical. The results for the individual Wilcoxon Signed-Rank test are presented in Table 6-6.

Table 6-6: Wilcoxon Signed-Rank Test Pairwise Comparison results for 6 x 3mm Q-Box™

Breast Quadrant	Comparison Quadrant	Median Difference in kPa	Positive Differences (N)	Negative Differences (N)	p-value
Lower Outer	Upper Outer	-1.69	28	16	0.037
	Upper Inner	-0.14	23	21	0.981
	Lower Inner	-0.60	22	22	0.506
Upper Outer	Upper Inner	2.41	13	31	0.043
	Lower Inner	1.53	16	28	0.126
Lower Inner	Upper Inner	0.32	21	23	0.912

These results suggest, when compared to the Lower Outer breast quadrant and the Upper Inner breast quadrants, the Upper Outer breast quadrant had a statistically significantly greater breast elasticity. The other quadrants had no significant differences.

The Final Analysis was using the CV to determine if there was a difference in the level of variability of elasticity data collected from each of the four quadrants. The results of the CV are presented in Table 6-7 below.

Table 6-7: Coefficient of Variation of the breast quadrants using the 6 x 3mm Q-Box™

Breast Quadrant	Coefficient of Variation (%)
Lower Outer	44.76
Upper Outer	41.35
Upper Inner	53.93
Lower Inner	50.76

From these results, it can be seen that there were some differences in the variability of the data; the CV was lowest for the Upper Outer and highest for the upper inner.

6.4.4.2 6 x 6mm Q-Box™

According to the Shapiro-Wilks analysis, the data showed to be not normally distributed. The Shapiro-Wilks results are shown in Table 6-8.

Table 6-8: 6 x 6mm Q-Box™ Shapiro-Wilk test for normality values

Quadrant	Shapiro-Wilk p-value
Lower Outer	0.080
Upper Outer	0.053
Upper Inner	0.001
Lower Inner	0.111

As the data was not normally distributed, again, a non-parametric test (Friedman Test) was used to analyse the data. The descriptive statistics are presented in Table 6-9.

Table 6-9: 6 x 3mm Q-Box™ descriptive statistics for comparison of values between breast quadrants

Quadrant	Median Elasticity (kPa)
Lower Outer	10.02
Upper Outer	11.85
Upper Inner	10.28
Lower Inner	10.38

The breast elasticity was statistically significantly different in the different breast quadrants $X^2(3) = 11.645$ and p-value of 0.009. The Wilcoxon Signed-Rank test was used for the pairwise comparison between the quadrants. The results from the Wilcoxon Signed-Rank test are presented in Table 6-10.

Table 6-10: Wilcoxon Signed-Rank Test Pairwise Comparison results for 6 x 6mm Q-Box™

Breast Quadrant	Comparison Quadrant	Median Difference in kPa	Positive Differences (n)	Negative Differences (n)	p-value
Lower Outer	Upper Outer	-1.75	30	14	0.056
	Upper Inner	-0.72	24	20	0.718
	Lower Inner	-0.57	24	20	0.653
Upper Outer	Upper Inner	2.44	13	31	0.080
	Lower Inner	1.98	14	30	0.071
Lower Inner	Upper Inner	-0.18	23	21	0.480

When using the 6 x 6mm Q-Box™, there were no statistically significant differences between any of the breast quadrants.

The Final Analysis was using the CV to determine if there was a difference in the level of variability of elasticity data collected from each of the four quadrants. The results of the CV are presented in Table 6-11 below.

Table 6-11: Coefficient of Variation of the breast quadrants using the 6 x 6mm Q-Box™

Breast Quadrant	Coefficient of Variation (%)
Lower Outer	45.71
Upper Outer	37.92
Upper Inner	49.14
Lower Inner	48.13

From these results, it can be seen that there were some differences in the variability of the data, as with the 6 x 3mm Q-Box™ elasticity data, the CV was lowest for the Upper Outer and highest for the Upper Inner.

6.4.4.3 Free-Trace Q-Box™

When using the Free-Trace Q-Box™, according to the Shapiro-Wilks analysis, the data were not normally distributed. The results from the Shapiro-Wilks analysis are presented in Table 6-12.

Table 6-12: Free-Trace Q-Box™ Shapiro-Wilk test for normality values

Quadrant	Shapiro-Wilk p-value
Lower Outer	0.059
Upper Outer	0.187
Upper Inner	0.012
Lower Inner	0.374

The descriptive results from the non-parametric Friedman Analysis are presented in Table 6-11.

Table 6-13: Free-Trace Q-Box™ descriptive statistics for comparison of values between breast quadrants

Quadrant	Median Elasticity in kPa
Lower Outer	9.88
Upper Outer	11.58
Upper Inner	10.28
Lower Inner	10.28

The Friedman test results were $X^2(3) = 3.144$ with a p-value of 0.370. This demonstrates that there were no significant differences between the breast quadrants when using the Free-Trace Q-Box™ protocol, and for this reason, the pairwise comparison does not need to be completed. This concludes that the group means were equal within this specific population when using this specific protocol.

The Final Analysis was using the CV to determine if there was a difference in the level of variability of elasticity data collected from each of the four quadrants. The results of the CV are presented in Table 6-14 below.

Table 6-14: Coefficient of Variation of the breast quadrants using the 6 x 6mm Q-Box™

Breast Quadrant	Coefficient of Variation (%)
Lower Outer	41.24
Upper Outer	32.12
Upper Inner	44.24
Lower Inner	42.94

From these results, it can be seen that there were some differences in the variability of the data, as with both the 6 x 3mm and 6 x 6mm Q-Box™ elasticity data, the CV was lowest for the Upper Outer and highest for the Upper Inner.

6.4.4.4 Comparison of Quadrants Summary

For all three protocols, the upper outer breast quadrant had the greatest elasticity. This was statistically significant when compared to the lower outer and the upper inner quadrants when using the three x 3mm Q-Box™ protocol. This quadrant also consistently had the lowest

CV, showing the least amount of variability of the collected data of the four quadrants. All other quadrants and protocols showed that the elasticity in the breast quadrants were equal.

6.4.5 Comparison of Left and Right Breast

The next analysis that needed to be completed was to determine if there was a difference between the left and right breast with the 6 x 3mm, 6 x 6mm and Free-Trace Q-Box™ data.

6.4.5.1 6 x 3mm Q-Box™

The initial analysis was to determine if the data was normally distributed. The data was tested for violation of normality using the Shapiro-Wilk test; the results suggest the data was not normally distributed for the left breast (p= 0.014) and was normally distributed for the right breast (p= 0.339).

Due to the non-parametric nature of the left breast data, the data were analysed using the Wilcoxon Signed-Rank test. All results are median values unless otherwise described. The descriptive statistics for the analysis are presented in Table 6-15.

Table 6-15: Descriptive statistics for 6 x 3mm Q-Box™ comparison of left and right breast

Left Breast	Right Breast	Difference
10.34 kPa	10.16 kPa	-0.22

The data of eleven participants with four repeat measure were used to determine if there was a difference in the elasticity values between the left and the right breasts as measured using the 6 x 3mm Q-Box™ protocol. The Wilcoxon Signed-rank test demonstrated there were no statistically significant median differences between the left and right breast, z=0.37, P=0.709.

Upon looking at the CV between the right and left breast, there were some differences when using the 6 x 3mm Q-Box™ protocol. The left breast had a CV of 45.90% and the right was 39.42%.

6.4.5.2 6 x 6mm Q-Box™

The initial analysis was to determine if the data were normally distributed. The data was tested for violation of normality using the Shapiro-Wilk test; the results suggest the data was normally distributed (left breast p-value = 0.0.051; right breast p-value = 0.195)

The paired samples T-test was used to analyse the data; the descriptive statistics are presented in table 6-17.

Table 6-16: Descriptive statistics for 6 x 6mm Q-Box™ comparison of left and right breast

Breast	Mean Elasticity in kPa (SD)
Left	11.29 (4.96)
Right	11.31 (4.22)

The mean difference was 0.015 (± 3.32) (95% CI -1.02 to 0.99; p=0.976). There were no statistically significant differences between the left and right breast when using the 6 x 6mm Q-Box™. Upon looking at the CV between the right and left breast, there were some differences when using the 6 x 6mm Q-Box™ protocol. The left breast had a CV of 43.99%, and the right was 37.28%.

6.4.5.3 Free-Trace Q-Box™

The initial analysis was to determine if the data was normally distributed. The data was tested for violation of normality using the Shapiro-Wilk test; the results suggested that the data was normally distributed (left breast p-value = 0.315; right breast p-value = 0.311). The paired samples T-test was used to analyse the data; the descriptive statistics are presented in Table 6-22.

Table 6-17: Descriptive statistics for Free-Trace Q-Box™ comparison of left and right breast

Breast	Mean Elasticity in kPa (SD)
Left	11.29 (4.97)
Right	11.31 (4.22)

The mean difference was -0.18 (±2.64) (95% CI -0.98 to 0.63; p=0.662). There are no statistically significant differences between the left and right breast when using the Free-Trace Q-Box™. Upon looking at the CV between the right and left breast, there were some

differences when using the Free-Trace Q-Box™. The left breast had a CV of 37.25% and the right breast was 34.58%.

6.4.5.4 Comparison of Left and Right Breast Summary

These analyses showed that with each of the three protocols, there were no differences between the left and right breasts. The CV showed some differences, with the right breast always having a lower CV but these differences may be arbitrary due to the singular data set and may have occurred due to chance.

6.5 Discussion

This study analysed the effect of using differing *post-hoc* SWE image analysis protocols on breast elasticity values. This analysis was undertaken to find the most precise method of measuring whole breast elasticity, as to date, there is no protocol to find the average whole breast elasticity; this being due to the novel and experimental nature of SWE being used for this indication. The data included in this study were re-analysed from the pharmacokinetic sub-study, which was presented in Chapter 4. This trial had recruited 11 participants who had four repeat SWE measurements, across a three-month time period. The Q-Box™ protocols that were used were as follows;

- 6 x 3mm Q-Box™ placed across the image
- 6 x 3mm Q-Box™ placed across the image
- A singular 10mm Q-Box™ placed in the middle of the image
- Full-Box trace Q-Box™, which was done using the Q-Box™ trace function to trace the border of the image
- Free-Trace Q-Box™, which was done using the Q-Box™ trace function and tracing the image to avoid all ultrasound artefacts and black holes present within the SWE image

In addition to these protocols, the Q-Box™ ratio function was used, this is when a singular Q-Box™ is placed on an area of glandular tissue, and another singular Q-Box™ is placed on an area of fatty tissue. This was done twice with some variation in the sizes of the Q-Box™ capturing the data for each of the tissue types. These additional protocols were used because

this is a commonly used protocol in previous research, and it needed to be determined if it is a reliable and valid method to find the whole breast elasticity.

As using whole SWE to measure whole breast elasticity is a novel biomarker for changes in the breast tissue, there is no gold standard of data collection methods in the literature to compare these SWE results to. This meant that there were some limitations in findings the protocol with the most valid results. The statistical model that was initially conducted was a one-way repeated measures ANOVA with *post-hoc* Bonferroni adjustments. The 6 x 3mm Q-Box™ was the reference standard for this analysis, as it was the original protocol used to collect the breast elasticity data. This statistical model was used to determine if any of the protocols produced elasticity values that were statistically similar or different to the 6 x 3mm Q-Box™ data. As there is no gold standard to provide the 'true' elasticity values, we can be more confident in the validity of the findings if multiple protocols produced similar results. When looking at the repeated measures ANOVA, if no other protocol produced similar data to the 6 x 3mm Q-Box™, it would have been reconsidered as the reference standard for this analysis. From the repeated measures ANOVA, the 6 x 6mm, Free-Trace, and alternative ratio Q-Box™ data showed no statistically significant differences in the acquired data. Bland-Altman plots were created to visually show the agreement and precision of the different Q-Box™ protocols when compared to the 6 x 3mm Q-Box™ data. Finally, the CV was calculated for all the datasets; this analysis presents a value that represents the dispersion of the data around the mean and can help determine the protocol with the most precise results.

From the statistical calculations and observations, it was found that the method with the greatest agreement and lowest amount of variation (lowest CV) was the Free-Trace Q-Box™ , which has previously been used in research by Evans (2015) for which the whole breast elasticity was also calculated using SWE. The other protocols which had similar data to the 6 x 3mm Q-Box™ data were the 6 x 6mm Q-Box™ and the alternative ratio data. The three main protocols that were considered to be used as the reference standard for future research were the 6 x 3mm Q-Box, 6 x 6mm Q-Box™ and Free-Trace Q-Box™ protocols. The Q-Box™ ratio data was omitted from this consideration, as although commonly used in scientific research as a method to calculate the elasticity of the glandular tissue in the breast, it did not appear to be a reliable method of breast elasticity quantification. This opinion is due to the fact that when using a singular Q-Box, the elasticity can be greatly influenced by where the Q-Box™ is

placed. As the elasticity differs throughout the image, one Q-Box™ is not a good representation of the whole breast quadrant. This misrepresentation was demonstrated within this study, as there were statistically significant differences in the average elasticity between the two ratio datasets. Using the Q-Box™ ratio function can also introduce large measurement bias to the study, as the operator can choose where the Q-Box™ is placed. This operator dependence and small ROI of an area of variable data potentially allows the elasticity to be manipulated by the researcher. This potential for manipulation can lead to unreliable data, especially in longitudinal studies, with repeat measurements, with the objective of determining the efficacy of an intervention or determining changes within the breast tissue.

As previously mentioned, the Free-Trace Q-Box™ was chosen as the protocol to use as the reference standard for future research. During this study, the Free-Trace Q-Box™ had the lowest CV; this showed that elasticity generated using this protocol had the greatest precision out of all the different protocols. The precision of a measurement shows the variability of the results and is a description of the random errors in the data. The Free-Trace Q-Box™ may have had the least variability due to the operator being able to omit objects within the image that are not true representations of the elasticity values. These objects include artefacts, which are areas on the colour elasticity image that might not be a representation of the mechanical properties of the breast tissue but rather an issue with the SWE. The areas in the image that have artefacts have extremely high elasticity values, which do not correspond to any structure on the B-Mode image (Figure 6-15)

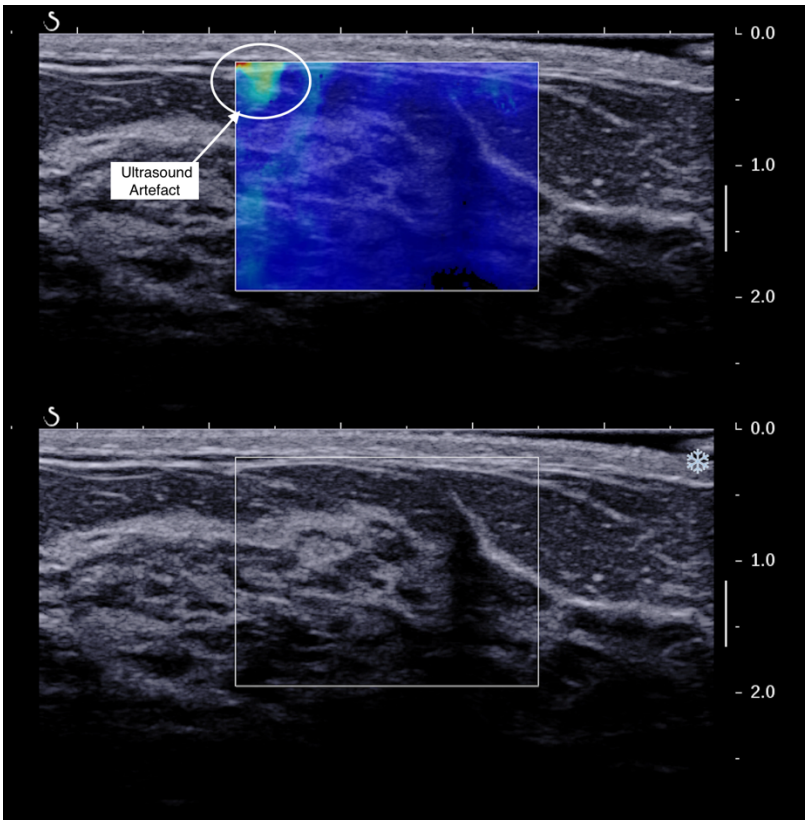

Figure 6-15: Shear wave Elastography image with Ultrasound artefact highlighted

The other objects that can induce random errors in the results are a phenomenon called 'black holes'; these are areas within the image for which the shear waves have not propagated. These black holes appear dark on the colour coded SWE output (Figure 6-16). As the shear waves have not propagated through the tissue, no elasticity values are recorded; consequently, the elasticity is displayed as 0.00kPa. With the Free-Trace Q-Box™, the operator can visualise, and trace around the artefacts and 'black holes', which decreases the random errors and variability in the data. This ability to trace around artefacts and 'black holes' increases the precision of the data when compared to the 6 x 3mm Q-Box™ data, as these can be placed around these areas but without as much precision, so may still include sections of 'black holes' or artefacts and lead to inaccurate elasticity data. As the Free-Trace Q-Box™ is still operator dependent, there is still the opportunity for operator bias to influence the elasticity values. However, as a larger ROI is included in the analysis, the actual placement of the box has less of an impact on the overall results.

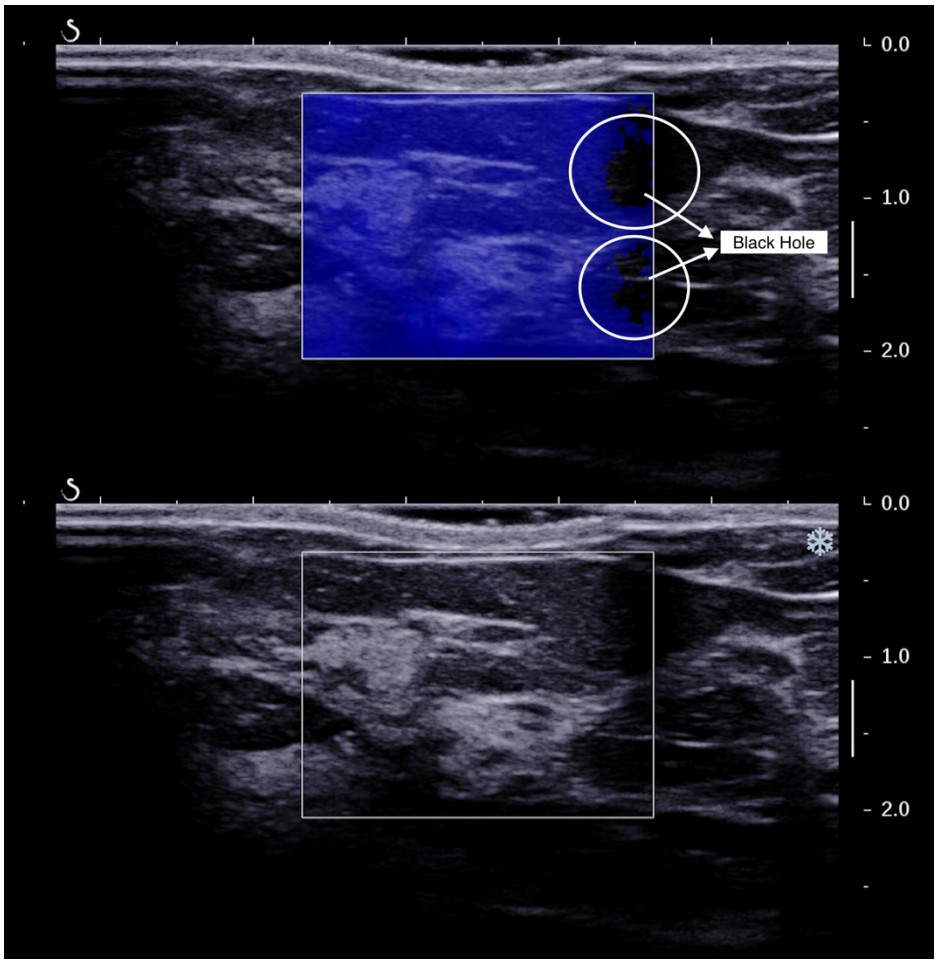

Figure 6-16: Shear Wave Elastography image with a 'black hole'; an area the shear waves have not propagated

Another reason for why the Free-Trace Q-Box™ was chosen as the future reference standard was its clinical utility. One of the key aspects of outcome measures that need to be analysed and considered, when being used for clinical and research purposes is the clinical utility. The clinical utility is how easy the outcome measure is to use, and the time taken to use the measure, this including the time taken to administer, enter the data and calculate the results. When using the SWE machine, manual analysis always needs to be conducted, as the process is not automated. The Free-Trace Q-Box™ is one of the quickest protocols studied with this analysis. For the protocols that require multiple Q-Box™, you are required to press the Q-Box™ button on the screen for each new Q-Box™ (Figure 6-17), size it using the roll-ball on the SWE machine (Figure 6-18) and then place it on the image in the desired location.

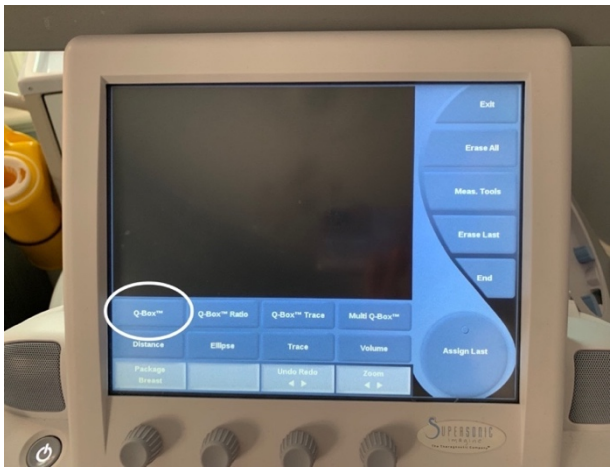

Figure 6-17: Shear Wave Elastography machine with the Q-Box™ function highlighted. This needs to be pressed six times when using the 3 x 6mm and 6 x 6mm protocols

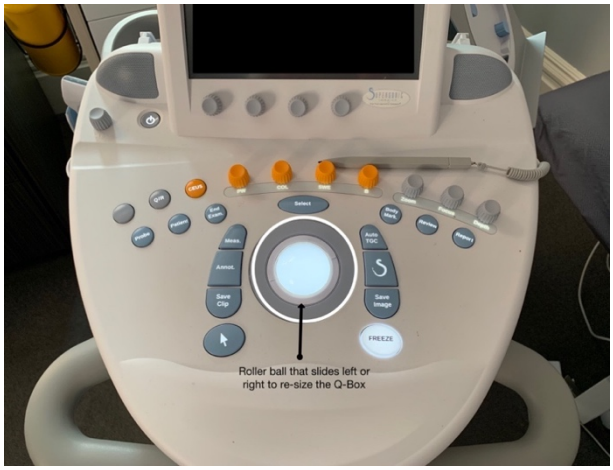

Figure 6-18: Shear Wave Elastography dial that can be slid from left to right to re-size the Q-Box™

Additionally, with the six Q-Box™, you then need to calculate the average elasticity from the six mean values for each Q-Box™ on the image. In contrast, when using the Free-Trace Q-Box™, the Q-Box™ command on the machine only needs to be pressed once, and then the operator proceeds to trace the ROI, avoiding the artefacts and black holes, using the attached stylus on the SWE machine (Figure 6-19). Only one set of data is also produced for each quadrant (Figure 6-20). This method generally only takes a few seconds per image. The clinical utility of the Free-Trace Q-Box™ is beneficial for larger clinical trials as the data can be generated in a timelier manner, there is less data that needs to be entered, and there is less need for manual calculations to determine the average elasticity of each quadrant.

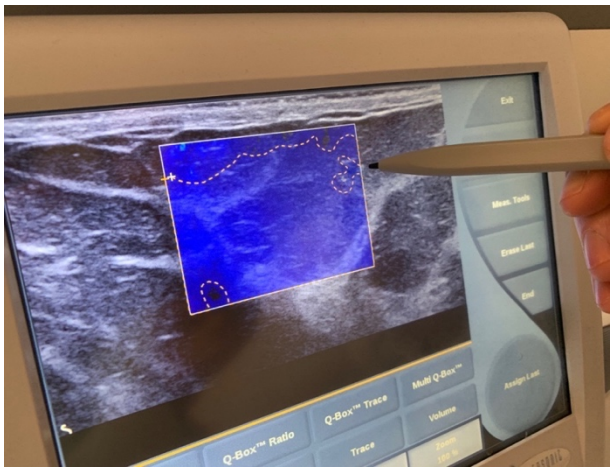

Figure 6-19: Using the attached stylus on the shear wave elastography machine to trace the region of interest using the Free-Trace Q-Box™ protocol

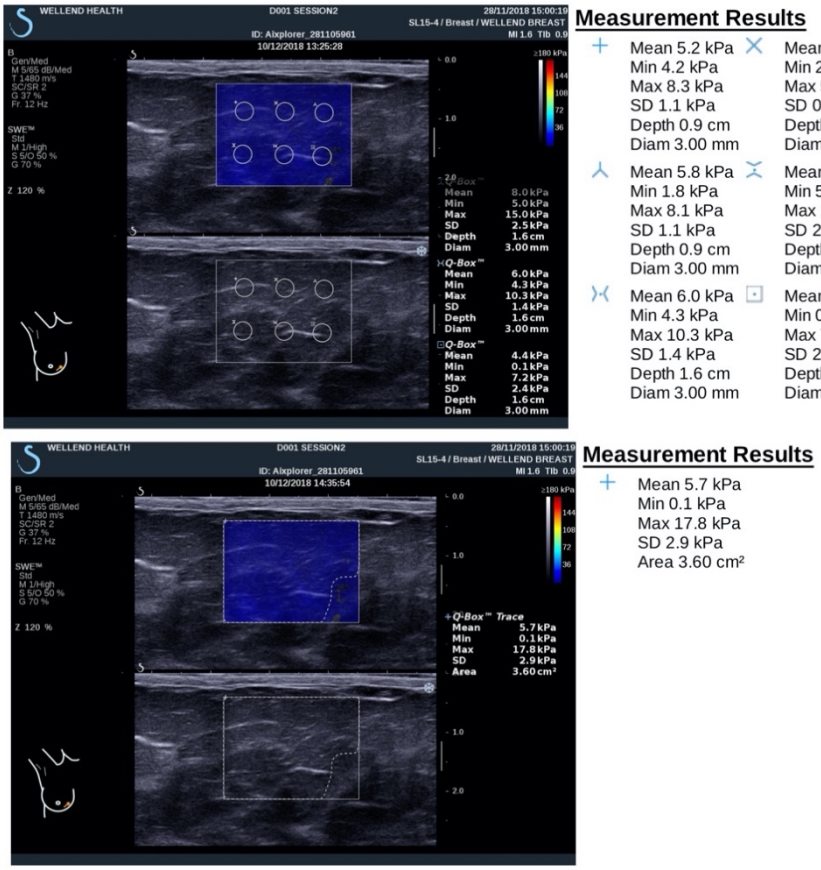

Figure 6-20: Comparison of the different shear wave elastography reports when using the 3 x 6mm Q-Box™ protocol and the Free-Trace Q-Box™ protocol

This study also analysed whether there were differences in the elasticity of the different breast quadrant, this was important as some studies analyse the data from just one quadrant and it is important that we know if there are inherent differences across the breast. The findings from this study suggest that when using all three of the protocols, the upper outer

breast quadrant consistently had a higher elasticity value. This data agreeing with pre-existing data, showing that the upper outer quadrant having the greatest dense area when compared to the other three quadrants (Chan, Chen et al. 2017), which may explain why the elasticity was increased in this area. When using the Free-Trace Q-Box™ method, there were no statistically significant differences in the breast elasticity. This showing that if a researcher wanted to make the SWE measure quicker, they could use a single breast quadrant if using the Free-Trace Q-Box™. The upper outer quadrant also consistently had the lowest CV, so if using just a single quadrant to measure the changes in the breast with repeat measures, it would be recommended to use the upper outer quadrant. These calculations, however, were based on a small sample size and with a repeat study may reveal that there are significant differences in the breast quadrants. Further research is required to make a definitive statement.

6.6 Conclusion

Using the SWE machine to measure the whole breast elasticity is a new indication for this device, and as such, there is limited research to guide the user towards the most valid and reliable method for collecting this data. This current study utilised a number of different protocols and found that the Free-Trace Q-Box™ protocol was the most precise method, with the lowest CV, and had one of the lowest levels of potential for the researcher or assessor to manipulate the data. These results and analyses into the different methods of elasticity data generation will aid future use of SWE in the research and clinical environment and can aid the increased consistency among different studies. As this was not the method used in the first two interventional studies in this research program, a re-analysis of the data of those studies is required to determine if the findings are significantly altered.

Chapter 7 Re-analysis of data from Chapter 4 and 6: utilising the Free-Trace Q-box™ function as described in Chapter 5.

7.1 Introduction

As described in both Chapter 4 and Chapter 5, the breast elasticity data showed a significant amount of within-subject variations; this variation did not appear to follow any particular trend or pattern. To further analyse this, the previous chapter (Chapter 6) described an analysis where the method of elasticity data generation was evaluated to see if this was an influence on the within-subject variation in the data. Seven different protocols were used, and from the analyses, it was determined that the method of using the Free-Trace Q-Box™ function had similar values to the 6 x 3mm Q-Box™ and 6 x 6mm Q-Box™ values. The Free-Trace Q-Box™ also had the lowest CV and therefore was the method which had the greatest precision. This level of precision was hypothesised to be due to the Free-Trace Q-Box™ having the largest area of the image included in in the ROI for which the elasticity is calculated for the quadrant. Also, when using the Free-Trace Q-Box™, the regions where the shear waves did not propagate, and the ultrasound artefacts that distort the elastic properties of the image, were omitted from the calculation. As the breast elasticity in Chapter 4 and Chapter 5 was collected using the 6 x 3mm Q-Box™ protocol, it was deemed to be of significant importance to repeat these analyses using the Free-Trace Q-Box™ to evaluate whether the initial results and conclusions are still valid.

7.2 Objective

The objective of this study was to re-analyse the data from Chapter 4 (HAVAH*T+Ai*™) and Chapter 5 (enobosarm and anastrozole) studies to determine if there were significant differences, which changed the findings and conclusions of the studies when using the Free-Trace Q-Box™ elasticity data, compared to the 6 x 3mm Q-Box™ elasticity data.

7.3 Method

The shear wave images that were taken for both the Chapter 4 (HAVAH*T+Ai*™) and Chapter 5 (enobosarm and anastrozole) studies were reopened on the SuperSonic™ Aixplorer® Shear Wave Ultrasound Machine. Each image for all the participants had the elasticity re-acquired using the Free-Trace Q-Box™ function. As per the previous description, the Free-Trace Q-Box™ function aimed to incorporate the largest ROI possible yet avoiding the areas that the shear waves have not propagated and any artefacts that may have distorted the average elasticity of the image.

Once the elasticity data for each image had been reacquired, reports were created for each participant visit and the data was entered into Microsoft Excel (2016). Following data entry, the same analyses that were initially conducted in each of the chapters, were redone with the data from the Free-Trace Q-Box™. The statistical analyses that were done for this chapter included;

- The comparison of the within-subject data of the different elasticity acquirement protocols for the HAVAH*T+Ai*™ study and the enobosarm and anastrozole study. If the data were normally distributed, it was analysed using a paired samples T-test. If not, the Wilcoxon-Sign tank test was used. If the distribution of the median difference was not symmetrical, the sign test was used to determine if there was a significant difference between the median values for each group.
- The repeat analysis of the change in elasticity over-time for the HAVAH*T+Ai*™ study and the enobosarm and anastrozole study. The initial analyses were conducted using the one-way repeat measures ANOVA if the data were normally distributed. If the data were not normally distributed, the Friedman test would be the statistical model chosen.
- The correlation between the elasticity values and mammography variable values for the HAVAH*T+Ai*™ study and Study two. This analysis was conducted using Pearson's correlation coefficient if the data were normally distributed. If the data was not normally distributed, a Spearman's rank-order correlation was used.

Statistical significance was set at $p \leq 0.05$ and with the confidence intervals for differences (where applicable) not crossing zero.

7.4 Results

7.4.1 Comparison of Within-subject Data - Pharmacodynamics (Breast Tissue Elasticity) of Combination Subcutaneous Testosterone and Anastrozole (HAVAH*T+Ai*™) in Premenopausal Women with High Mammographic Breast Density

The first analysis that was completed had the objective of determining if there was a statistically significant difference between the within-subject data points of the Free-Trace Q-Box™ values and the 6 x 3mm Q-Box™ values. The breast elasticity from each participant, at each time point, for both of the variables were used in this analysis. The difference score was computed, the data were checked for outliers, and the Shapiro-Wilk test was used to assess the normality of the data. The p-value for the Shapiro-Wilk test was 0.000, which established that the data was not normally distributed; from visually inspecting the data on a histogram, it was observed that there was a strong positive skew in the dataset. From visually inspecting a box-plot of the data, it was observed that there were two outliers present in the dataset. These outliers were assessed and were deemed to be true values and were included in the analysis. Upon looking at the symmetry of the median differences, it was observed that the data was not symmetrical. Therefore, the Sign Test was the statistical model chosen to analyse the data. The data are all reported as median values unless otherwise stated; the descriptive statistics are presented in Table 7.1.

Table 7-1: Descriptive statistics for the comparison of Q-Box™ protocols of within-subjects from the pharmacodynamic (breast tissue elasticity) of combination subcutaneous Testosterone (T) and anastrozole (Ai) in premenopausal women with high MBD

6 x 3mm Q-Box™ Median Elasticity	Free-Trace Q-Box™ Median Elasticity	Difference
10.39kPa	10.14kPa	-0.11kPa

There was a total of 44 data-points included in the analysis; 25 of these had positive differences, 19 had negative differences, and there were zero ties within the differences. The

results from the Sign Test show that there were no statistically significant median differences between the 6 x 3mm Q-Box™ data and the Free-Trace Q-Box™ data, z= 0.754, p= 0.451.

7.4.1.1 Summary

These results showed that when using the Free-Trace Q-Box™ protocol, there were no differences in the elasticity values that were obtained using the original 6 x 3mm Q-Box™ protocols.

7.4.2 Repeat Analyses - Pharmacodynamics (Breast Tissue Elasticity) of Combination Subcutaneous Testosterone and Anastrozole (HAVAH*T+Ai*™*)* in Premenopausal Women with High Mammographic Breast Density

7.4.2.1 Change in Breast Elasticity Over Time with Repeat Measurements

The following analysis was evaluating the change in the breast elasticity data, at different time points, with the repeat measurements. This analysis was done to calculate if there were changes in the breast elasticity across the study using the Free-Trace Q-Box™ data, and whether this outcome differed from the original calculations using the 6 x 3mm Q-Box™ elasticity data. As with the analysis above, initially, the data was assessed using the Shapiro-Wilk test. The Shapiro-Wilk test results are presented in Table 7-2.

Table 7-2: Shapiro-Wilk values for the repeat measure analysis using the Free-Trace Q-Box™ elasticity

Measurement	Shapiro-Wilk p-value
Baseline	0.052
Month 1	0.001
Month 2	0.000
Month 3	0.001

The Shapiro-Wilk test result suggests that the data was not normally distributed. Using the boxplot to visually assess the data, five outliers were observed within the data; three outliers were in the one-month dataset, one was in the second-month dataset, and one was in the third-month dataset. These outliers were checked and were deemed to be true values and were to be included in the analysis. As the data was non-parametric, the Friedman test was used to analyse the data.

7.4.2.1.1 Friedman Test of Free-Trace Q-Box™ Data

The median values for the Friedman test are presented in Table 7-3.

*Table 7-3: Median values for each time point using the Free-Trace Q-Box™ for the repeat analysis of pharmacodynamics (breast tour elasticity) of combination subcutaneous Testosterone (T) and anastrozole (Ai) (*HAVAH*T+Ai™) in premenopausal women with high MBD*

Measurement	**Median Elasticity (kPa)**	**Difference from Baseline (kPa)**
Baseline	12.20	
Month 1	10.65	-1.55
Month 2	8.50	-3.70
Month 3	7.85	-4.35

The results from the Friedman test demonstrated that the breast elasticity values were statistically significantly different at the different time points during the time period that the women were on the HAVAH*T+Ai*™ intervention, X^2 = 27.734, p<0.0005. The pairwise comparisons from the Friedman test are presented in Table 7-4.

Table 7-4: Results of the pairwise comparison of the Friedman test with comparisons from baseline to the different time points and adjusted significance values

Timepoint	**Adjusted Significance**
Baseline to Month 1	1.000
Baseline to Month 2	0.002
Baseline to Month 3	<0.0005

The results from the pairwise comparison component of the Friedman test demonstrate that the breast elasticity had statistically significant reductions from baseline to month two, and from baseline to month three.

7.4.2.1.2 Original Results from Chapter 4

The original results of the one-way repeat measure ANOVA using the 6 x 3mm Q-Box™ data are presented in table 7-5.

Table 7-5: Original results using the 6 x 3mm Q-Box™ for the one-way repeat measures ANOVA

Timepoint	Breast Elasticity (SD) in kPa	Change from Baseline (kPa)	95% CI	p-value
Baseline	13.67 (7.89)			
Month 1	11.68 (5.58)	-1.99	-4.35 to 0.36	0.148
Month 2	9.86 (5.63)	-3.80	-6.72 to -0.87	0.004
Month 3	8.63 (3.96)	-5.04	-7.31 to -2.78	>0.005

These two results demonstrate that when using the Free-Trace Q-Box™, the results are not substantially different from the original calculations using the 6 x 3mm Q-Box™ data. The original figures showed greater decreases over the three months of the study, however both reductions at month two and month three reached statistical significance, and both of the p-values were similar between the two different data collection protocols.

7.4.2.2 Correlations Between Breast Elasticity and Per Cent Volumetric Breast Density Using the Free-Trace Q-Box™ Elasticity Data

The next section of analyses is the correlations between the breast elasticity data and the %VBD data. The first analysis was the correlation between the baseline %VBD and baseline breast elasticity.

7.4.2.2.1 Baseline Breast Elasticity and Baseline Per Cent Volumetric Breast Density

The Shapiro-Wilk test was again used to determine the normality within the data. The normality of the data was not violated from the results of this test (baseline elasticity p-value = 0.128; baseline %VBD p-value 0.286). For this reason, Pearson's correlation coefficient was the statistical model chosen to perform the analysis. Using Pearson's correlation coefficient, the results produced an *r*-value of 0.252 (p=0.456); this is a negligible correlation between the two variables (Figure 7-1).

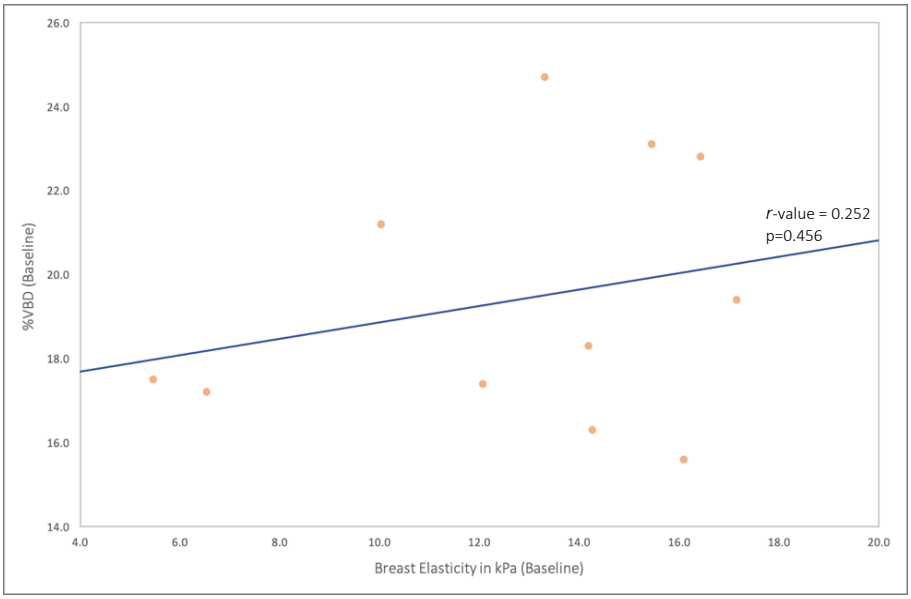

Figure 7-1: Correlation between baseline %VBD and baseline breast elasticity in kPa

The original 6 x 3mm Q-Box™ data produced an *r*-value of 0.184 (p=0.589). This result was a negligible correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data did not change the overall outcome of the correlation analysis.

7.4.2.2.2 End of Study Breast Elasticity and End of Study Per Cent Volumetric Breast Density

The next analysis that was conducted was to analyse the correlations between the EOS %VBD data and the EOS breast elasticity data. The Shapiro-Wilk test was again used to determine normality within the data. The results showed that the data was normally distributed (EOS elasticity p-value = 0.559; EOS %VBD p-value = 0.320). For this reason, the Pearson's correlation coefficient was the statistical model chosen for the analysis. The results showed an *r*-value of 0.256 (p=0.448), which is a negligible correlation between the two variables. The scatterplot with the trendline for this correlation is presented in Figure 7-2.

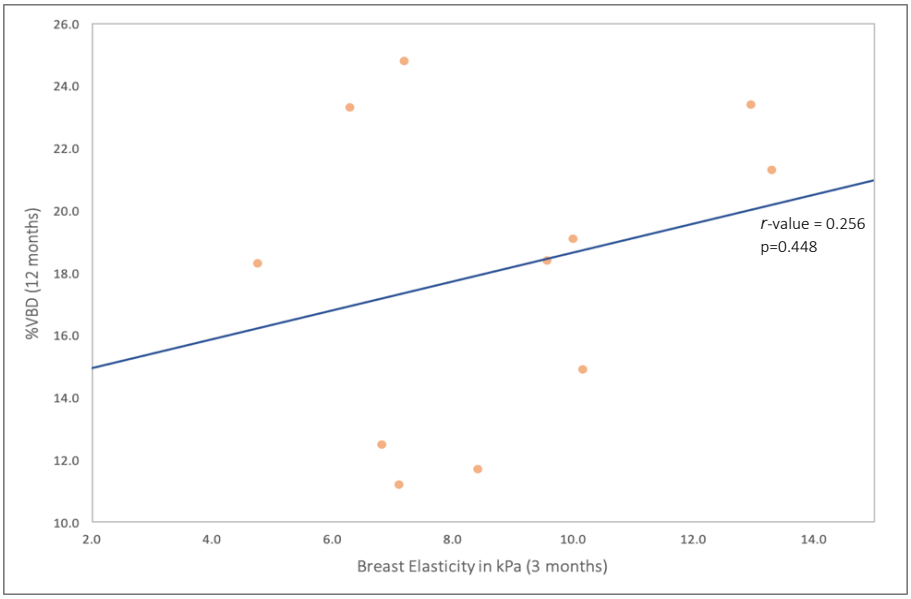

Figure 7-2: Correlation between EOS %VBD and breast elasticity at 3 months (final Breast elasticity measurement)

The original correlation coefficient using the 6 x 3mm Q-Box™ data produced an *r*-value of 0.233 (p=0.491), which is also a negligible correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data did not change the overall outcome of the correlation analysis.

7.4.2.2.3 Changes in Breast Elasticity and Changes in Per Cent Volumetric Breast Density

When determining if there was a correlation between the changes in %VBD and the change in the breast elasticity values for the Free-Trace Q-Box™ data, the same analyses that were originally conducted in Chapter 4 were completed. The initial correlation was on the difference values between the baseline %VBD and the 12-month %VBD results, and the difference values between the baseline elasticity and the three-month elasticity findings. The Shapiro-Wilk test results suggested the data was normally distributed (change in elasticity (baseline to 3 months) p-value = 0.099; change in %VBD (baseline to 12 months) p-value = 0.287). For this reason, the Pearson's correlation coefficient was used to analyse the data. The results show an *r*-value of 0.234 (p = 0.488), which showed a negligible correlation between the two variables. The scatterplot with the trendline for this correlation is presented in Figure 7-3.

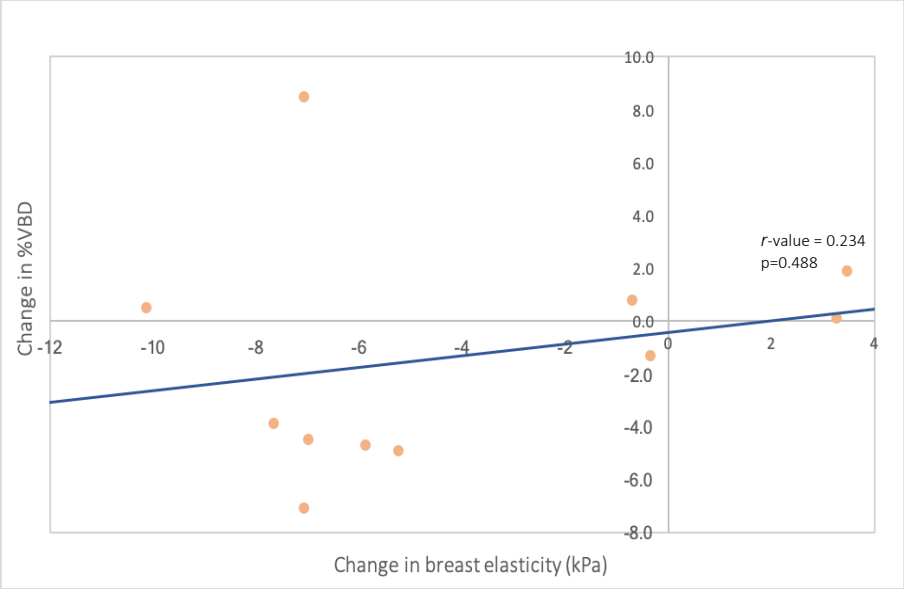

Figure 7-3: Correlation between change in %VBD (baseline to month 12) and breast elasticity in kPa (baseline to month 3)

The original calculation using the 6 x 3mm Q-Box™ data had a similar finding with an *r*-value of 0.315 (p=0.345), which was a low positive correlation between the variables across the study. Using the Free-Trace Q-Box™ elasticity data did slightly alter the outcome of the correlation analysis, as it went from low to a negligible correlation. Both findings are clinically insignificant, as it does not show a valuable relationship between the two variables.

7.4.2.2.4 Summary

These results show that there were some differences in the individual correlation coefficients, but these differences did not change the overall outcomes and conclusions of the analyses. The findings using the Free-Trace Q-Box™ data suggest that there were negligible correlations between %VBD and breast elasticity, and the changes in %VBD and changes in elasticity.

7.4.2.3 Correlations Between Elasticity and Total Fibroglandular Volume

The next group of analyses was the correlations between the TFV measurements, and the breast elasticity values and the changes in these variables.

7.4.2.3.1 Correlation Analysis Between Baseline Total Fibroglandular Volume and Breast Elasticity

The initial analysis was to determine if there was a correlation between the baseline TFV and the baseline breast elasticity. The Shapiro-Wilk test results suggest the baseline elasticity was normally distributed (p=0.128), and the baseline TFV data violated normality (p=0.014). As one of the variables was not normally distributed, the Spearman's rank-order correlation coefficient was the statistical model chosen to analyse the data. Spearman's coefficient produced an *r*-value of 0.100 (P=0.770). This was a negligible correlation between the baseline TFV and the baseline breast elasticity (Figure 7-4).

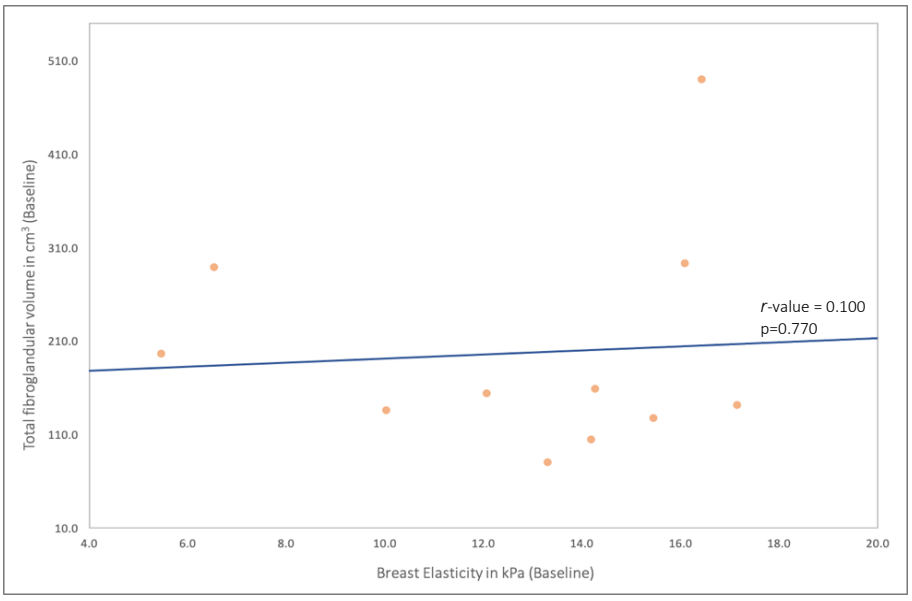

Figure 7-4: Correlation between total fibroglandular volume at baseline and breast elasticity at baseline

This result was a similar result to the original analysis using the 6 x 3mm Q-Box™ data which produced an *r*-value of -0.188 (p=0.729). Using the Free-Trace Q-Box™ elasticity data did not change the overall outcome of the correlation analysis, except one was a positive and one was a negligible correlation.

7.4.2.3.2 End of Study Total Fibroglandular Volume and End of Study Breast Elasticity

The next analysis was conducted to determine the correlation between the EOS TFV, which was measured at 12 months and the EOS breast elasticity, which was measured at 3 months. The Shapiro-Wilk test results suggest the EOS elasticity is normally distributed (p-value = 0.559), and the EOS TFV data violated normality (p-value = 0.035). Again, the Spearman's rank-order correlation coefficient was used to analyse the data. The Spearman's coefficient produced an r^S value of -0.427 (p=0.190); this demonstrates a low correlation between the two variables (Figure 7-5).

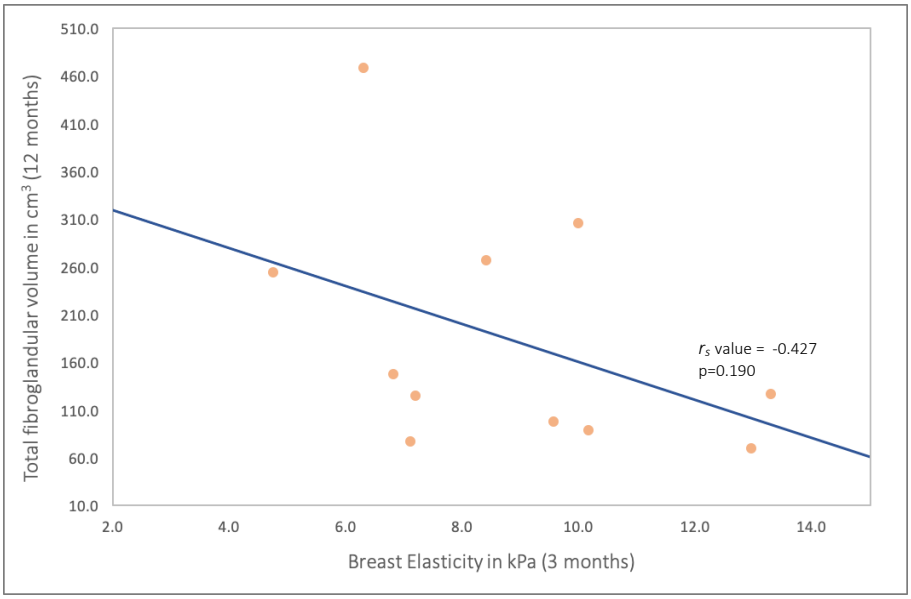

Figure 7-5: Correlation between EOS total fibroglandular volume (12 month) and EOS Breast Elasticity (3 months)

The original analyses using the 6 x 3mm Q-Box™ data, the Spearman's rank-order correlation produced a r^S value of -0.436 (p=0.180), which again was a low, negative correlation between the variables. Using the Free-Trace Q-Box™ elasticity data did not alter or change the overall outcome of the correlation analysis.

7.4.2.3.3 Correlation Between Change in Total Fibroglandular Volume and Change in Breast Elasticity

The next analysis is a correlation between the change in TFV from baseline to month 12 and the change in breast elasticity from baseline to month three. The Shapiro-Wilk test results suggest that both sets of the data were normally distributed (change in elasticity (baseline to 3 months) p-value = 0.099; change in TFV (baseline to 12 months) p-value = 0.104). As the data was parametric, the Pearson's correlation coefficient was the statistical model chosen, and this analysis produced and *r*-value of 0.616 (p=0.044), this was a statistically significant moderate correlation between the two variables (Figure 7-6).

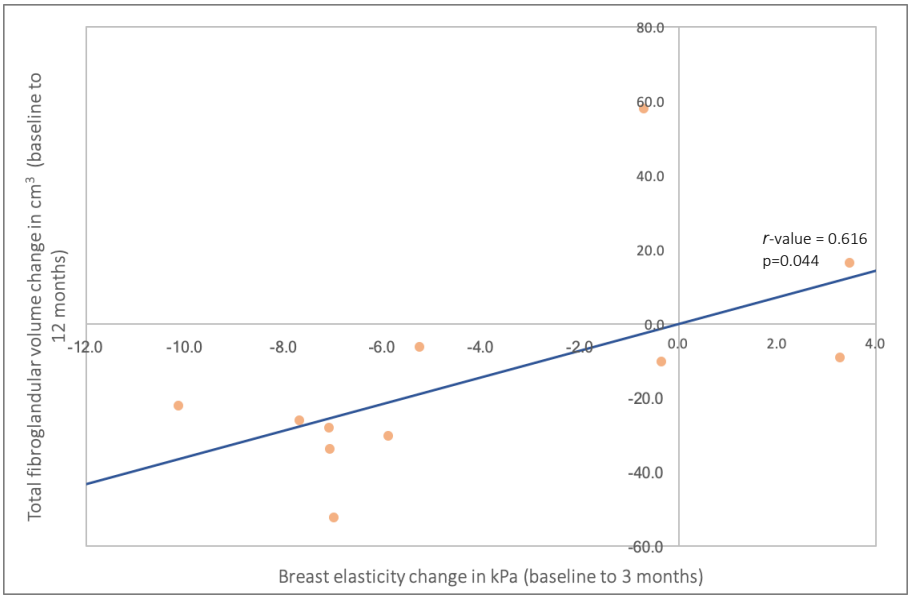

Figure 7-6: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 3)

In regard to the previous analysis conducted in Chapter 4, Pearson's correlation coefficient demonstrated that the *r*-value was 0.689 (p=0.019). This result again was a statistically significant moderate positive correlation between the change in elasticity from baseline to month three and the change in TFV from baseline to month 12. Using the Free-Trace Q-Box™ elasticity data did not change the outcome of the correlation analysis.

Further to this analysis, a correlation analysis was conducted on the change in TFV between baseline and month 12 and the change in elasticity from baseline to month one and the

change in breast elasticity from baseline to month two. The Shapiro-Wilk test suggests that the change in elasticity (baseline to 1 month) data violated normality (p-value = 0.042), and the change in elasticity (baseline to 2 months) was normally distributed (p-value = 0.558). As the change in elasticity from baseline to month one was not normally distributed; the Spearman rank-order correlation was used to analyse this data. The change in breast elasticity from baseline to month two was normally distributed, and for this reason, Pearson's correlation coefficient was used to analyse the data.

Firstly, the Spearman rank-order correlation produced a r^s value of 0.609 (p=0.047) between the change in TFV and the change in breast elasticity from baseline to one month. This result was a statistically significant moderate correlation between the two variables (Figure 7-7).

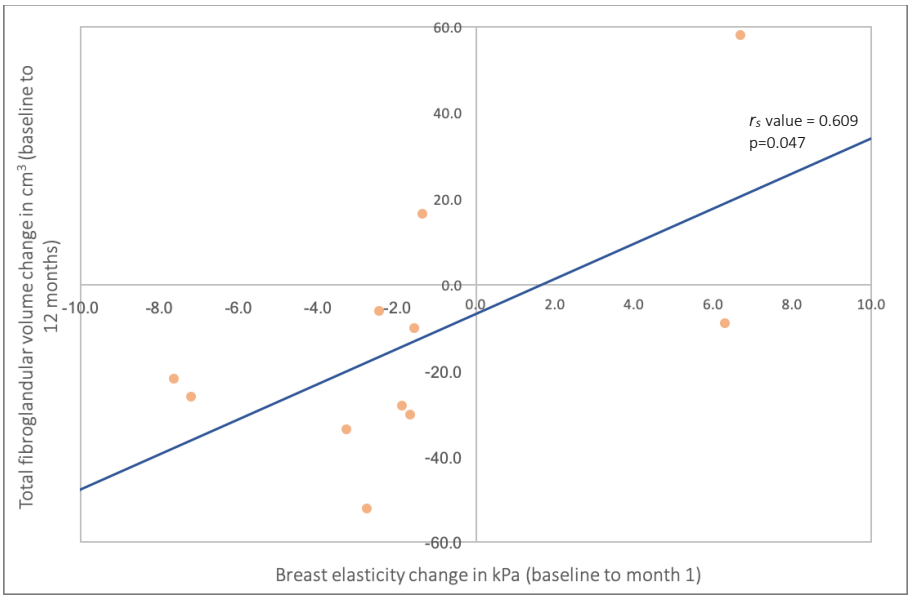

Figure 7-7: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 1)

The original analysis that was conducted in Chapter 4 using the 6 x 3mm Q-Box™ produced an *r*-value of 0.611 (p=0.046), which again was a statistically significant moderate positive correlation between the elasticity change at month one and the TFV change at 12 months. Using the Free-Trace Q-Box™ data did not change the overall outcome of the correlation analysis.

The last analysis was the correlation between the TFV change and the elasticity change from baseline to month two. Pearson's correlation coefficient produced an *r*-value of 0.813

(p=0.002) (Figure 7-8). This result was a statistically significant, high correlation between the two variables.

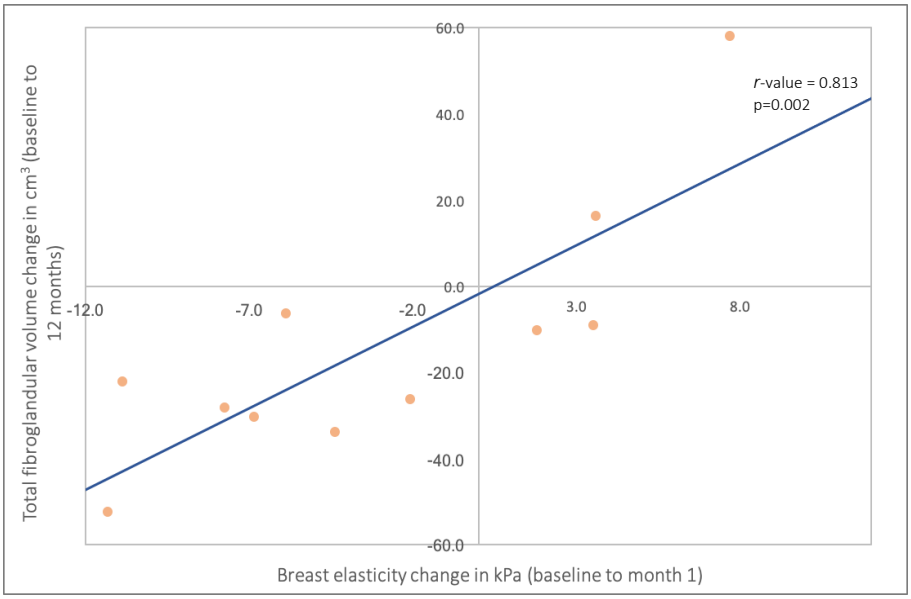

Figure 7-8: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 2)

The original analysis that was conducted in Chapter 4 using the 6 x 3mm Q-Box™ produced very similar results with an *r*-value of 0.818 (p=0.002), which was a statistically, high correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data did not change the overall outcome of the correlation analysis.

7.4.2.3.4 Summary

These analyses again showed that there were no significant differences in the outcomes between the original results using the 6 x 3mm Q-Box™ data and the Free-Trace Q-Box™ elasticity data. The conclusions remain that there are moderate to strong correlation between the changes in TFV and changes in breast elasticity. Also, the same conclusions remain that there is a moderate and strong correlation with early breast elasticity changes seen at one month and month two, respectively, with the changes in TFV across 12 months. These results may not have changed significantly with the Free-Trace Q-Box™ data as, although the method of data collection has a lower CV and it less influenced by the artefacts and black holes, the actual spread of the data has remained the same, while the mean elasticity is lower. This would conclude the correlations remained mostly the same, yet the data used was different.

These results show that breast elasticity may be able to predict early the changes occurring in the potential changes in TFV at 12 months, which gives promise for breast elasticity being a biomarker for changes within the breast tissue with further research.

7.4.3 Comparison of Within-subject Data - Anastrozole and GTx-024: The Effect of an Aromatase Inhibitor and Selective Androgen Receptor Modulator on Mammographic Breast Density and Breast Elasticity in Premenopausal Women

The initial analysis that was conducted in this section was to determine if there was a statistically significant difference between the within-subject data points, using the Free-Trace Q-Box™ and 6 x 3mm Q-Box™ data. Upon looking at the normality of the data, the Shapiro-Wilk test showed a significance value of 0.000, which demonstrated that the data was not normally distributed, and there was a strong negative skew with the data. There was one extreme outlier that sat greater than 1.5 box lengths from the edge of the boxplot. Upon further analysis, this figure was deemed a correct figure and kept within the analysis. The Sign test was the statistical model chosen as the distribution of the median differences were not symmetrical. All data are reported as median values unless otherwise reported. The descriptive statistics are presented in Table 7-6.

Table 7-6: Descriptive statistics for the within-subjects data from anastrozole and GTx-024: the effect of an aromatase inhibitor and selective androgen receptor modulator on MBD and breast elasticity in premenopausal women

6 x 3mm Q-Box™ Median Elasticity	Free-Trace Q-Box™ Median Elasticity	Difference
14.05 kPa	13.77 kPa	-0.70 kPa

There was a total of 39 data points within the analysis, 10 had positive differences, 29 negative differences, and there were zero ties within the differences. The Sign test demonstrated that there was a statistically significant median difference of -0.70kPa, the standardized test statistic (z) = -2.88 and a p-value of 0.004.

7.4.4 Repeat Analyses - Anastrozole and GTx-024: The Effect of an Aromatase Inhibitor and Selective Androgen Receptor Modulator on Mammographic Breast Density and Breast Elasticity in Premenopausal Women

7.4.4.1 Change in Elasticity Over Time with Repeat Measurements

This analysis is evaluating the repeat measures of the Free-Trace Q-Box™ breast elasticity data. This analysis was conducted to determine if there was a statistically significant difference in the elasticity data at the different time points and whether this differed from the original analyses using the 6 x 3mm Q-Box™.

The data were first analysed to establish the normality of the data and whether there were any outliers in the data. The results from the Shapiro-Wilk test are presented in Table 7-7.

Table 7-7: Shapiro-Wilk test for normality for the Free-Trace repeat measures data

Measurement	Shapiro-Wilk Value
Baseline	0.032
Month 1	0.321
Month 3	0.048
Month 12	0.148

The baseline and month three datasets were both not normally distributed; month one and month 12 were normally distributed. There were also two outliers present in the data; these were in the month one and month three datasets. Upon further inspection, these data points were deemed true values and were left to be included in the analysis. As not all of the data were normally distributed, the Friedman test was the statistical model used to analyse the data. All values are median values unless otherwise stated. The median values for the breast elasticity values are presented in Table 7-8.

Table 7-8: Descriptive statistics for the Free-Trace Q-Box™ data

Timepoint	Median Elasticity (kPa)	Difference in Elasticity from Baseline (kPa)
Baseline	15.00	
Month 1	13.35	-1.65
Month 3	11.50	-3.50
Month 12	13.40	-1.60

The Friedman test showed that the breast elasticity was statistically significantly different at the different time points throughout the study $X^2(3) = 10.762$, p=0.013. The pairwise comparisons are presented in Table 7-9.

Table 7-9: Results from the Friedman test pairwise comparison for Free-Trace Q-Box™ data with adjusted statistical significance

Timepoint	Adjusted Significance p-value
Baseline to Month 1	1.000
Baseline to Month 3	0.014
Baseline to Month 12	0.094

These results show that there was a statistically significant decrease in the median breast elasticity from baseline to Month 3 however there was a slight increase in the median elasticity values from month 3 to month 12, resulting in statistically insignificant changes from baseline to month 12.

7.4.4.1.1 Original Results from the 6 x 3mm Q-Box™ One-way Repeat Measures ANOVA

The original one-way repeat measures ANOVA using the 6 x 3mm Q-Box™ results are presented in Table 7-10.

Table 7-10: Original one-way repeat measure ANOVA using 6 x 3mm Q-Box™

	Value (SD)	Change from Baseline (SD)	95% CI	p-value
Breast Tissue Elasticity (kPa)				
Baseline	17.94 (9.35)			
Month 1	14.25 (4.76)	-3.69 (9.83)	-7.50 to 0.12	0.064
Month 3	13.94 (7.75)	-4.00 (11.18)	-8.36 to 0.364	0.097
Month 12	12.55 (5.34)	-5.37 (11.54)	-9.85 to -0.92	0.009

The original results which used the 6 x 3mm Q-Box™ presented more dramatic decreases in the breast elasticity across the four repeat measurements. The results showed a trend for decreasing elasticity values across the three-time points after baseline, with no mean elasticity increases at any time point. The decreases reached statistical significance at the 12-month dataset.

7.4.4.1.2 Summary

These results demonstrate that when using the Free-Trace Q-Box™ data, there are significant differences in the outcomes and conclusions regarding the data. The elasticity decreased from baseline in both the datasets; however, when using the Free-Trace Q-Box™ data, the elasticity increased slightly at the 12-month repeat measure. This result could demonstrate that the breast elasticity has quite rapid acute decreases and then begins to plateau as the timeline extends. Further explanations for the discrepancies between the results produced by the two elasticity protocols could be that the 6 x 3mm Q-Box™ data is more influenced by artefacts and fluctuations in the data and it may have been possible that artefacts were included in the baseline images, increasing the mean elasticity and showing greater decreases with the follow-up measurements. In addition to this, there may have been areas in the month 12 images where the shear waves didn't propagate (black holes), which produces a lower mean elasticity and therefore may show greater reductions with time. Furthermore, as the elasticity fluctuates across each SWE image and the 6 x 3mm Q-Box™ method requires the operator to place the Q-Box™ across the image, it is possible that by chance the Q-Box™ were placed on areas on lower elasticity, thus producing a lower mean elasticity at the 12-month time point.

Further research needs to be completed to determine if there is a true elasticity plateau at some point during the intervention, or to determine if there were measurement errors at any point during the study.

7.4.4.2 Correlations Between Elasticity and Per Cent Volumetric Breast Density

The next group of analyses that were conducted were the analyses of the correlation between the %VBD and the breast elasticity values.

7.4.4.2.1 Correlation Between Baseline Per Cent Volumetric Breast Density and Breast Elasticity

The initial calculation conducted was to determine the correlation between the baseline %VBD and the baseline breast elasticity values. The Shapiro-Wilk suggested that the data was normally distributed (baseline elasticity p-value = 0.778; baseline %VBD p-value = 0.185). As the data was normally distributed the Pearson's correlation coefficient was the statistical model used the analyse the data. The results from the Pearson's correlation coefficient showed there was no correlation between the two variables with an *r*-value of 0.000 (p=0.999) (Figure 7-9). There was no pattern of a relationship between baseline breast elasticity and baseline %VBD.

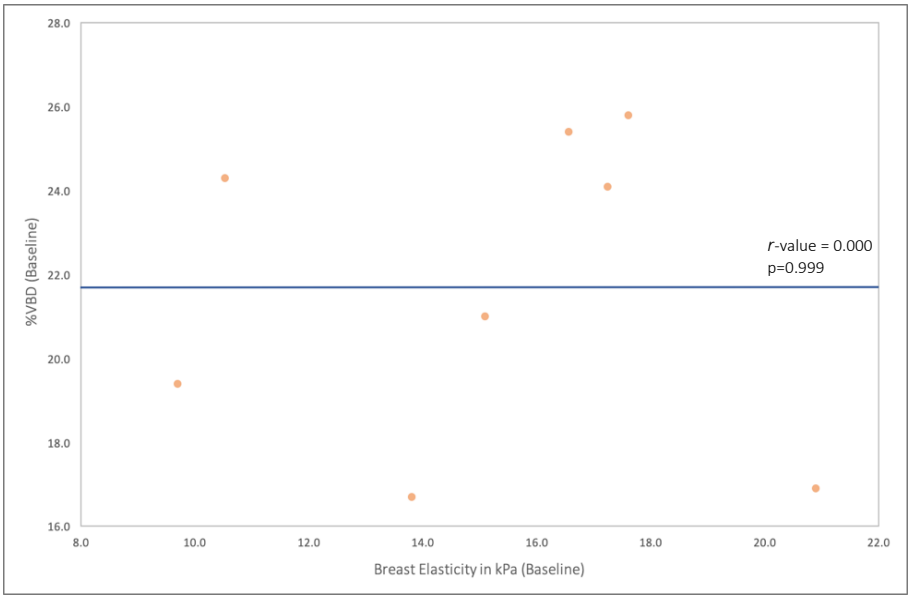

Figure 7-9: Correlation between %VBD at baseline and breast elasticity in kPa at baseline

The original results conducted in Chapter 5 using the 6 x 3mm Q-Box™ data. These correlations resulted in an *r*-value of 0.264 (p=0.528), which was a negligible correlation between the two variables. Using the Free-Trace Q-Box™ data did not change the result of the correlation analysis.

7.4.4.2.2 Correlation Between End of Study Per Cent Volumetric Breast Density and End of Study Breast Elasticity

The next analysis was to correlation the EOS %VBD values with the EOS breast elasticity values. The Shapiro-Wilk results suggested both sets of data were normally distributed (EOS elasticity p-value = 0.596; EOS %VBD p-value = 0.576).The Pearson's correlation coefficient produced an *r*-value of 0.114 (p=0.788); this was a negligible correlation between the two variables (Figure 7-10).

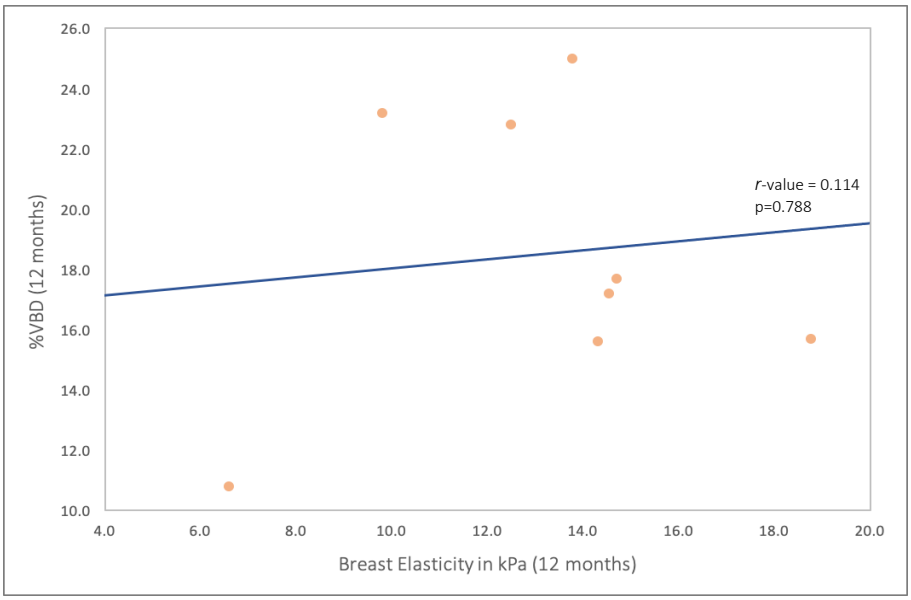

Figure 7-10: Correlation between EOS %VBD (12-month data) and EOS breast elasticity (12 months)

This result was similar to the original analysis presented in Chapter 5, using the 6 x 3mm Q-Box™ data. This analysis produced an *r*-value of 0.273 (p=0.523), which was a negligible correlation between the two variables. Using the Free-Trace Q-Box™ data did not change the overall result of the correlation analysis.

7.4.4.2.3 Correlation Between the Change in Per Cent Volumetric Breast Density and Change in Breast Elasticity

The initial calculation was looking at the correlation between the change in breast elasticity and the change in %VBD from baseline to the final 12-month repeat measurement. The data was normally distributed as per the Shapiro-Wilk test (change in elasticity (baseline to 12 months) p-value = 0.609; change in %VBD (baseline to 12 months) p-value = 0.435). The results from Pearson's correlation coefficient produced an *r*-value of 0.315 (p=0.448), which was a low correlation between the two variables (Figure 7-11)

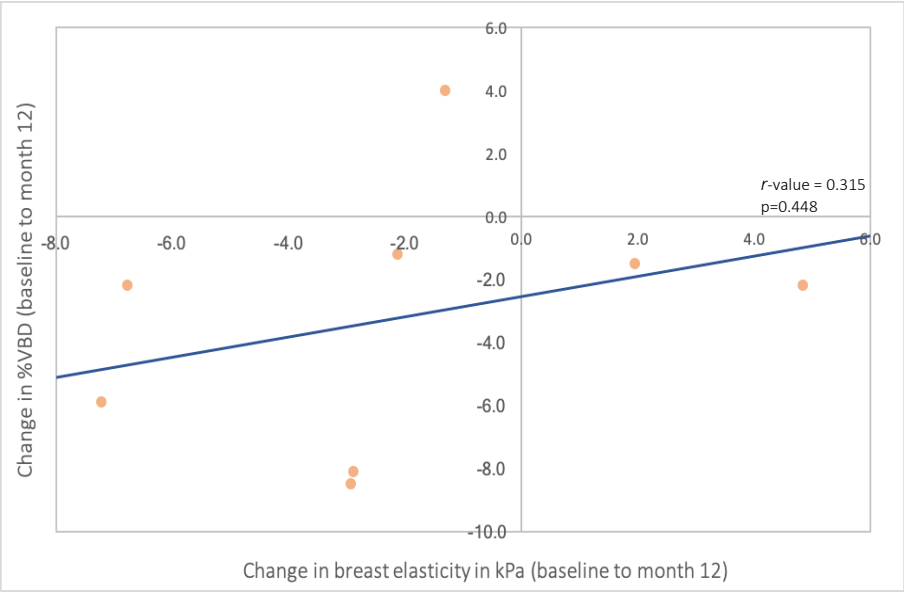

Figure 7-11: Correlation between change in %VBD (baseline to month 12) and change in breast elasticity (baseline to month 12)

These results differed from the original analysis, which used the 6 x 3mm Q-Box™ data. These analyses produced an *r*-value of -0.120 (p=0.997), which was a negligible, negative correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data changed the correlation from negligible to low.

The final analysis for this section was using the Free-Trace Q-Box™ data to correlate the changes in %VBD from baseline to the 12-month measurements, and the change in elasticity from the baseline measurement to the one-month time point. The data was normally distributed according to the Shapiro-Wilk test (change in elasticity (baseline to one month) p-value = 0.231; change in %VBD (baseline to 12 months) p -value = 0.435). The Pearson's

correlation coefficient produced an *r*-value of 0.041 (p=0.925); this was a negligible, positive correlation between the two variables (Figure 7-12).

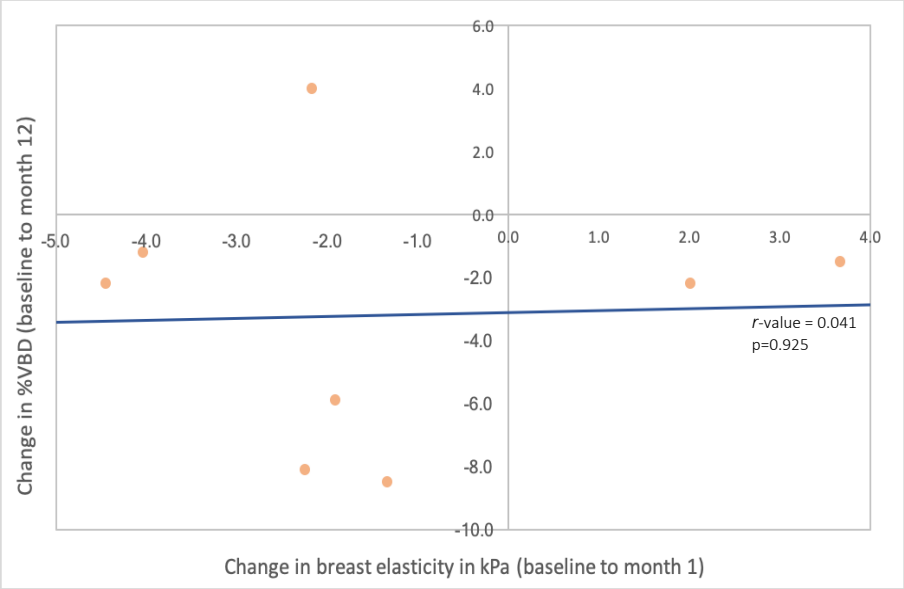

Figure 7-12: Correlation between change in %VBD (baseline to month 12) and change in breast elasticity (baseline to month one)

These results vary significantly from the original analysis using the 6 x 3mm Q-Box™ data, presented in Chapter 5. This analysis produced an *r*-value of -0.451 (p=0.262), which was a low negative correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data changed the correlation analysis from moderate to negligible.

7.4.4.3 Correlations Between Elasticity and Total Fibroglandular Volume

The next group of analyses is correlation the breast elasticity values with the TFV values.

7.4.4.3.1 Correlation Between Baseline Breast Elasticity and Baseline Total Fibroglandular Volume

The initial analysis was to correlate the baseline elasticity values with the baseline TFV values. The data was normally distributed as per the Shapiro-Wilk test (baseline elasticity p-value = 0.779; baseline TFV p-value = 0.264). The Pearson's correlation coefficient was used to analyse the data. The correlation coefficient produced an *r*-value of 0.078 (p=0.854); this a negligible, positive correlation between the two variables (Figure 7-13).

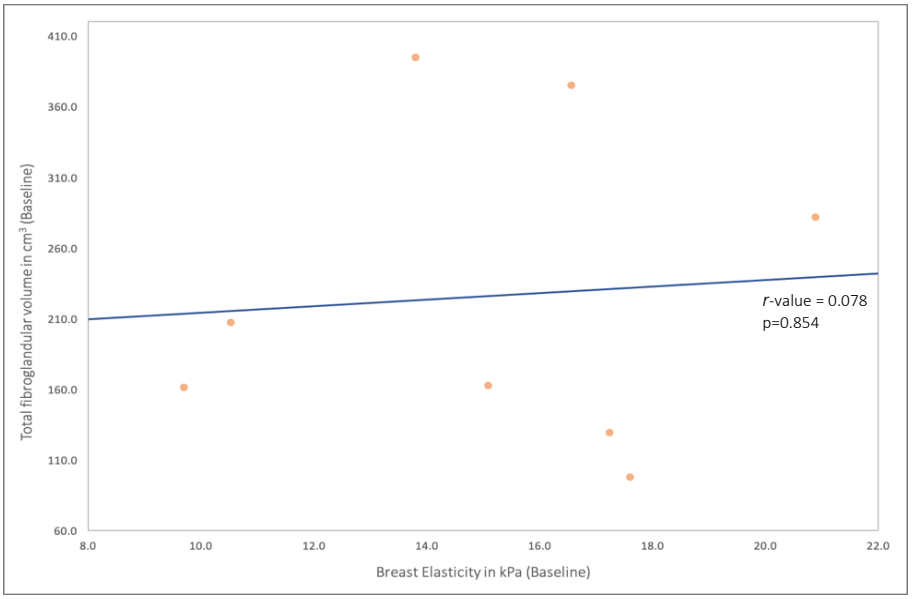

Figure 7-13: Correlation between baseline total fibroglandular volume and baseline breast elasticity

The original calculation produced a similar result to the one just presented. The original analysis produced an *r*-value of 0.273 (p=0.523), which was a negligible, positive correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data changed the correlation analysis outcome from low to negligible.

7.4.4.3.2 Correlation Between End of Study Breast Elasticity and End of Study Total Fibroglandular Volume

The next correlation conducted was the correlation between the EOS breast elasticity and the EOS TFV. The data was normally distributed as per the Shapiro-Wilk test (EOS elasticity p-value = 0.596; EOS TFV p-value = 0.462). The Pearson's correlation coefficient was the statistical test used. The results of this test produced an *r*-value of -0.547 (p=0.161), which was a negative, moderate correlation between the two variables (Figure 7-14).

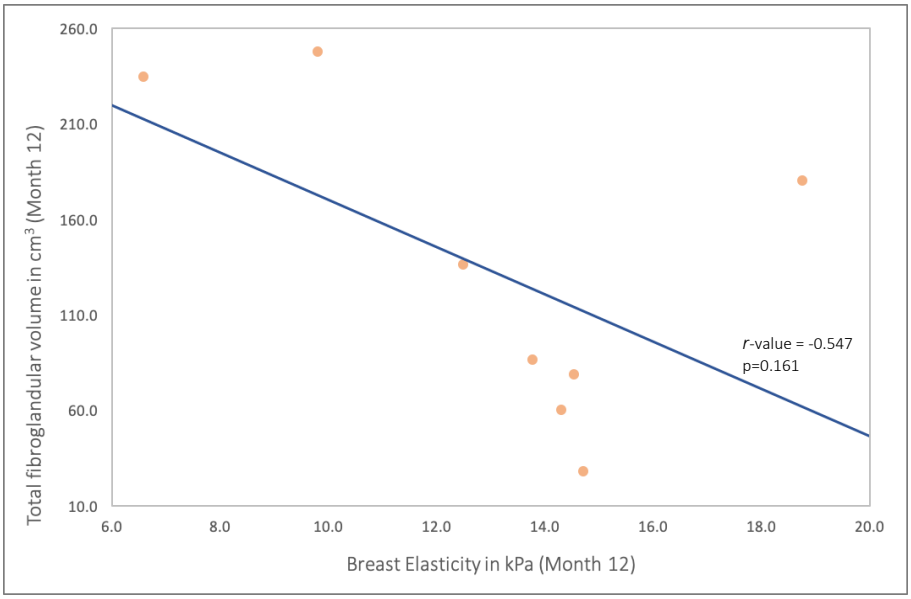

Figure 7-14: Correlation between EOS total fibroglandular volume (12 months) and EOS breast elasticity (12 months)

The original result presented in Chapter 5 was substantially different from the results just presented. The results from Chapter 6 showed an *r*-value of 0.842 (p=0.009), which was a statistically significant, high positive correlation between the two variables. Using the Free-Trace Q-Box™ elasticity data changed the results from a positive to a negative correlation and changed the overall correlation from high to moderate.

7.4.4.3.3 Correlation Between the Change in Breast Elasticity with the Change in Total Fibroglandular Volume

The first analysis in this group of analyses is a correlation the change in elasticity from the baseline measure to the 12-month time point with the change in TFV from the baseline measure to the 12-month time point. According to the Shapiro-Wilk test the TFV violated the test for normality (p=0.034), and therefore the data were transformed using a logarithmic transformation. Following the data transformation, the Shapiro-Wilk test demonstrated the change in TFV was 0.087, which was normally distributed. The change in elasticity was normally distributed (p = 0.609). As both datasets were now normally distributed, Pearson's correlation coefficient was used to analyse the data. This correlation analysis produced an *r*-value of 0.657 (p=0.077); this was a moderate, positive correlation between the two variables (Figure 7-15).

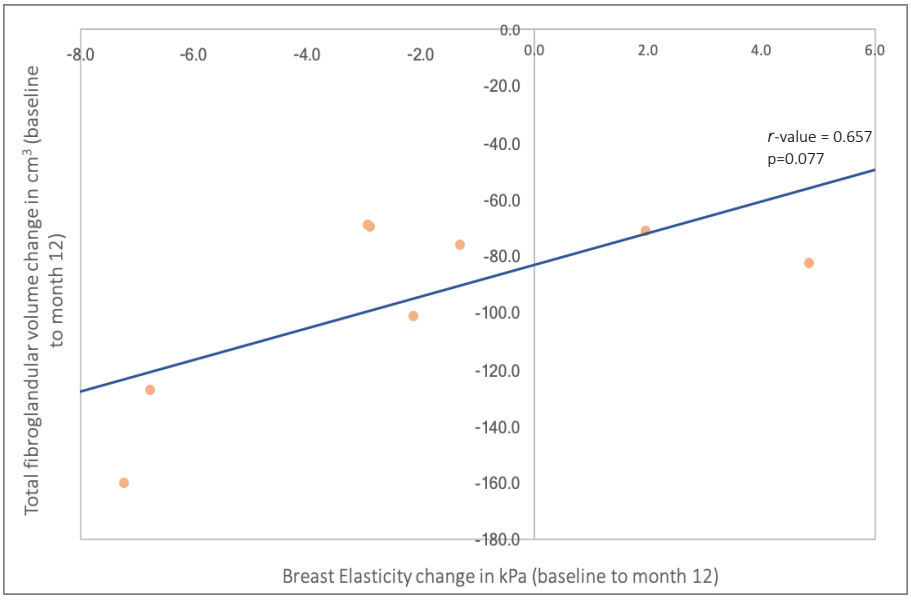

Figure 7-15: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 12)

The original results in Chapter 5 produced a similar result with an *r*-value of 0.678 (p=0.139), which again was a moderate correlation between the two variables. Using the Free-Trace Q-Box™ data did not change the overall conclusion from the correlation analysis.

Upon evaluating the change in breast elasticity from baseline to one month, and its correlation to the changes in TFV from baseline to month 12. The results from the Shapiro-Wilk test showed the change in elasticity (baseline to one month) was normally distributed (p=0.231). The change in TFV was listed above and was normally distributed with transformed data. The results from the Pearson's correlation coefficient produced an *r*-value of 0.459 (p=0.253), which was a positive, low correlation between the two variables (Figure 7-16).

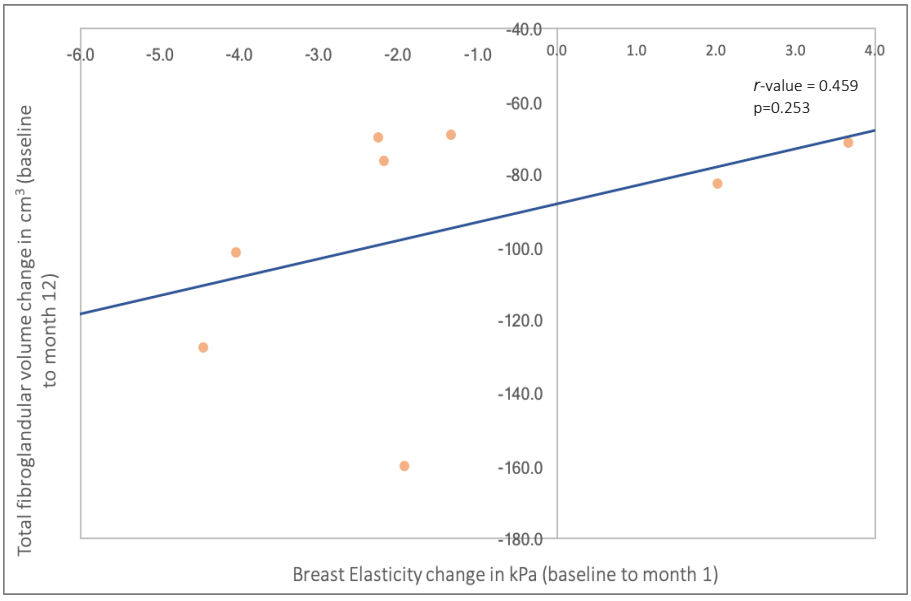

Figure 7-16: Correlation between change in total fibroglandular volume (baseline to month 12) and change in breast elasticity (baseline to month 1)

This result was a similar result to the original analysis in Chapter 5 with an *r*-value of 0.500 (p=0.313), which was a positive, moderate correlation between the two variables. Using the Free-Trace Q-Box™ data changed the overall correlation from moderate to low; however, the actual *r*-values are quite similar in value.

7.4.4.3.4 Correlation Between Elasticity and Total Breast Volume

The next group of analyses is between the breast elasticity and the TBV at the different time points.

7.4.4.3.5 Correlation Between Baseline Breast Elasticity and Baseline Total Breast Volume

The Initial analysis conducted was the correlation between the baseline elasticity and the baseline TBV. The data was normally distributed according to the Shapiro-Wilk test (baseline elasticity p-value 0.778; baseline TBV p-value = 0.345), as the data was normally distributed the Pearson's correlation coefficient was used to analyse the data. This correlation produced an *r*-value for this correlation was 0.095 (p=0.822), which was a positive, negligible correlation between the two variables (Figure 7-17).

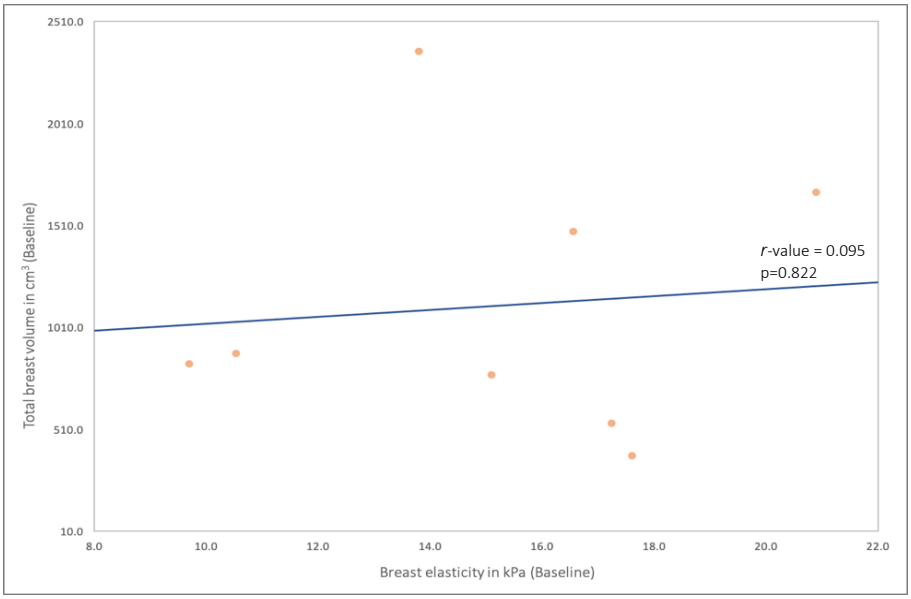

Figure 7-17: Correlation between baseline total breast volume and baseline breast elasticity

This result was a similar result to the original results presented in Chapter 5, with this correlation producing an *r*-value of 0.246 (p=0.557), which again was a positive, negligible correlation between the two variables. Using the Free-Trace Q-Box™ data did not alter the conclusion from the correlation analysis.

7.4.4.3.6 Correlation Between End of Study Breast Elasticity and End of Study Total Breast Volume

Upon looking at the EOS elasticity and the EOS TBV, the data was normally distributed (EOS elasticity p-value = 0.596; EOS TBV p-value = 0.086). Pearson's *r*-value was -0.605 (p=0.112), which was a negative, moderate correlation between the two variables (Figure 7-18).

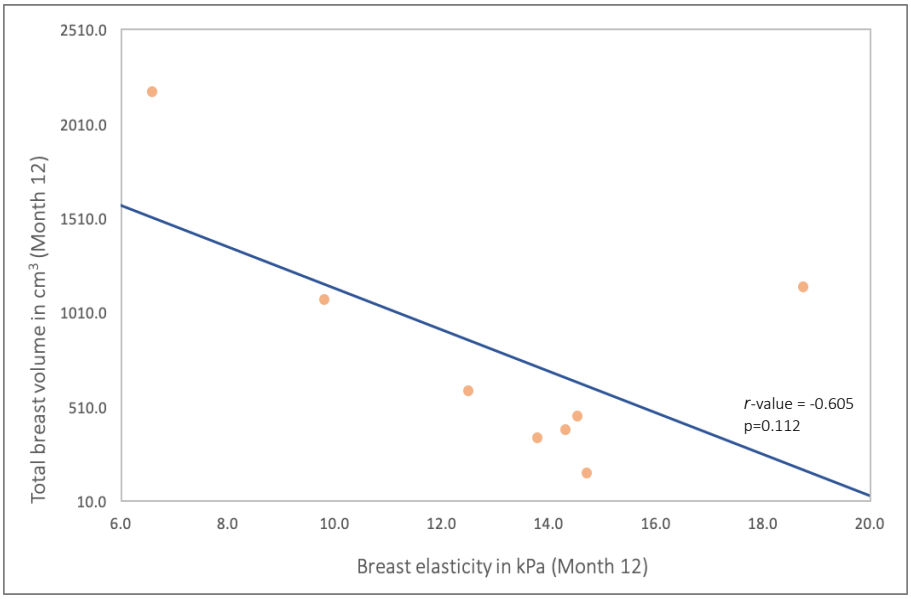

Figure 7-18: Correlation between EOS total breast volume (12 months) and EOS breast elasticity (12 months)

These results differed substantially from the original analysis presented in Chapter 5; this analysis produced an *r*-value of 0.845 (p=0.007), this being a statistically significant strong correlation between the two variables. Using the Free-Trace Q-Box™ data, the results went from a positive, negligible relationship to a negative high positive correlation between the two variables.

7.4.4.3.7 Change in Breast Elasticity and Change in Total Breast Volume

The next analysis was to look at the correlation between the change in breast elasticity from baseline to 12 months and the change in TBV from baseline to 12 months. The data was normally distributed according to the Shapiro-Wilk test (change in elasticity (baseline to 12 months) p-value = 0.623; change in TBV (baseline to 12 months) p-value = 0.735). The Pearson's *r*-value was -0.225 (p=0.592). This result was a similar result to the previous analysis that was presented in Chapter 5 with that analysis producing an *r*-value of 0.280 (p=0.502), both correlations were statistically insignificant, low correlations between the two variables (one a positive, one a negative correlation) (Figure 7-19).

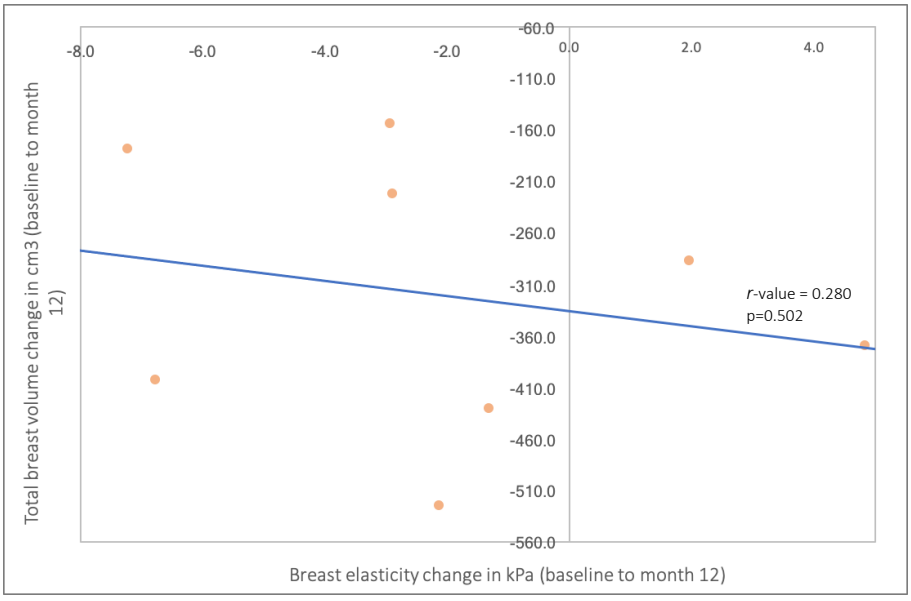

Figure 7-19: Correlation between change in total breast volume (baseline to month 12) and change in breast elasticity (baseline to month 12)

The final analysis was to correlation the change in elasticity from baseline to month one and the change in TBV from baseline to month 12. Again, the data was normally distributed according the Shapiro-Wilk test (change in elasticity (baseline to one month) p-value = 0.231), and the change in TBV was listed above. The Pearson's correlation coefficient produced an *r*-value of 0.283 (p=0.497), which was a low, positive correlation between the two variables (Figure 7-20). This result was different from the original analysis, which resulted in an *r*-value of 0.585 (p=0.223), which was a positive, moderate correlation between the two variables. Therefore, using the Free-Trace Q-Box™ elasticity data changed the overall conclusion of the correlation analysis.

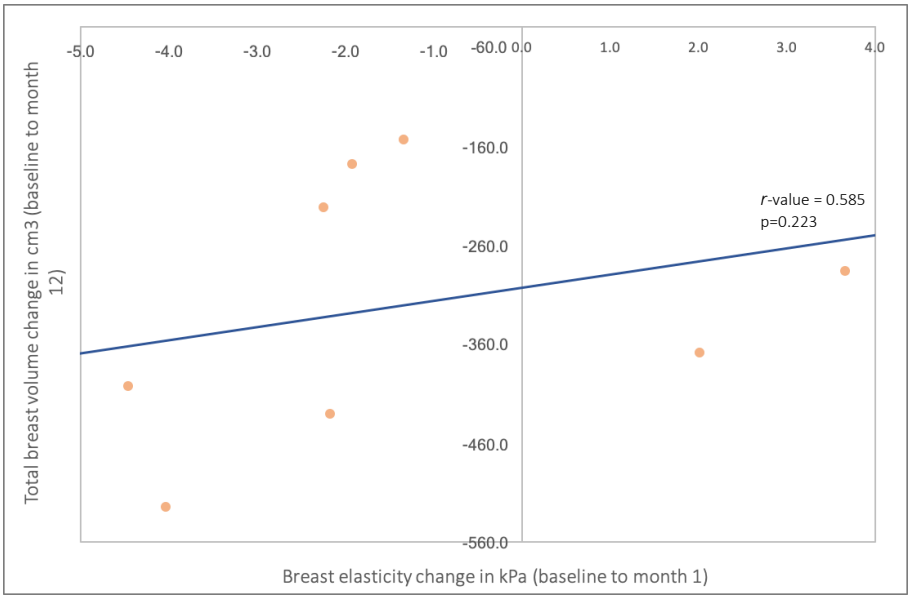

Figure 7-20: Correlation between change in total breast volume (baseline to month 12) and change in breast elasticity (baseline to month one)

7.5 Discussion

Within the original experimental studies located in Chapter 4 (HAVAH*T+Ai*™ study) and Chapter 5 (enobosarm and anastrozole study) of this thesis, the whole breast elasticity was calculated from SWE images using 6 x 3mm Q-Box™ across eight images (each breast quadrant) and then averaging the elasticity from these Q-Box™. Observations of the data in these studies revealed substantial within-subject variation, which did not appear to follow a pattern or a consistent trend of increasing or decreasing breast elasticity. Due to these observations, it was decided that the elasticity data needed to be examined, and that the data would be re-acquired using a variety of different Q-Box™ protocols to extract the data from the SWE image. Collecting this data was done to evaluate which methods had high levels of agreement with the reference standard, and which method had the lowest CV, therefore the greatest precision when collecting the elasticity data. In addition, the clinical utility of the data collection method was evaluated, as for this research program the focus is the use of SWE in clinical research, therefore the chosen SWE method needed to be easily implemented in research protocols and be able to be conducted in a timely manner.

The best protocol for collecting the data was determined to be the Free-Trace Q-Box™ method, which is the method of tracing the ROI over the image; omitting any areas where the

shear waves have not propagated and omitting areas where artefacts have corrupted the image. When using this method, firstly, there isn't the same degree of elasticity variation due to the areas of extremely high and low elasticity being omitted as per the method stated above. Additionally, when using this method, there is a large area to calculate the average elasticity, which provides a more accurate representation of the assumed true elasticity from the image. When using the 6 x 3mm Q-Box™ protocol, a substantial amount of area in the SWE image is not included in the elasticity calculation. As the elasticity in the tissue fluctuates to such a great extent, smaller ROIs may substantially alter the value for the whole breast elasticity. To determine if changing the method of analysing the SWE images influenced the results of the original two experimental studies, the original images were re-evaluated using the Free-Trace Q-Box™, and all the relevant analyses that were conducted in these studies were repeated with the new dataset to determine if the outcomes differed.

Regarding the within-subject data of the HAVAH*T+Ai™* study of Chapter 4, there were no statistically significant median differences between the two elasticity datasets (6 x 3mm and Free-Trace Q-Box™). However, when analysing the within-subject data of the enobosarm and anastrozole study of Chapter 5, there were statistically significant median differences between the two datasets. The Free-Trace Q-Box™ had a slightly lower median elasticity of 13.77kPa which was 0.7kPa lower than that produced with the 6 x 3mm Q-Box™ method. As mentioned in Chapter 6, using different Q-Box™ protocols or sizes and varying the position the Q-Box™ are placed can alter the elasticity output, and this was demonstrated within this analysis. From this analysis it can be reinforced that it is crucial that a consistent elasticity protocol is used amongst researchers to generate the mean whole breast elasticity or measurement error may bias the results, increasing the risk of Type 1 errors (false positives).

Regarding the repeat measurements and the analysis of the change in elasticity over time, in the HAVAHT*+Ai*™ study the original data and subsequent analyses using the 6 x 3mm Q-Box™ protocol, the results showed statistically significant decreases in the mean breast elasticity from the second month with reductions of -3.80kPa (95% CI -6.72 to 0.87;p=0.0004), and there was also a statistically significant reduction at the three-month time point with a decrease of -5.04kPa (95% CI -7.31 to -2.78; p<0.005). When using the Free-Trace Q-Box™ data in the second analysis, the trends of decreasing elasticity were similar, but the overall

decreases were slightly less than the original analysis. In the Free-Trace Q-Box™ analysis, as with the original analyses, there were statistically significant reductions at month two of -2.97kPa (95% CI -5.30 to -0.64; p=0.005) and at month three with reduction of -4.04kPa (95% CI -5.84 to -2.24; p<0.0005). Both datasets showed a statistically significant reduction at the same time points; the Free-Trace Q-Box™ data may have had a smaller reduction due to the decreased variability of the data. As the method decreases the potential for elasticity extremes, it may have led to observed smaller mean changes. The Free-Trace Q-Box™ data also had smaller confidence intervals, which demonstrates a greater level of precision of the results. This smaller confidence interval being favourable for clinical research and shows a greater indication of efficacy in studies with smaller sample sizes. Overall, these results demonstrate that the original conclusions that HAVAH*T+Ai*™ influenced breast elasticity can be maintained.

Similar findings were observed in the repeated measure analysis of the enobosarm and anastrozole study from Chapter 5. The original analysis demonstrated that there were statistically significant changes in the breast elasticity at the 12-month time point with a change of -5.37kPa (95% CI -9.85 to -0.92; p=0.009). The breast elasticity changes also approached statistical significance at the one-month time point with a change of -3.69kPa (95% CI -7.50 to 0.12; p=0.064). When using the Free-Trace Q-Box™, as the data was non-parametric, the Friedman test and median values were used. This analysis showed a statistically significant reduction at the third month with a change of -3.50kPa (p=0.014) and the 12-month reductions plateaued, leading to a statistically insignificant change of -1.60kPa (p=0.094). The changes between month three and month 12 were statistically insignificant with a breast elasticity increase of 0.58kPa (95% CI -1.58 to 2.69; p=1.000). If the Free-Trace Q-Box™ elasticity analysis was redone using a parametric test, as per the Friedman test, there were statistically significant changes of -2.76kPa (95% CI -5.39 to -0.12; p=0.094) at the third month time point. These results then plateaued and became statistically insignificant at the 12-month time point with a reduction of -2.18kPa (95% CI -4.57 to 0.209; p=0.094). One of the major limitations of this study is that we do not know if this was a true plateau with the tissue elasticity or whether there was a measurement error. A future study with more regular SWE imaging may be able to provide more insight into what is happening with the breast elasticity between the third and 12th month.

With the Free-Trace Q-Box™ data, even though the results were not as favourable for the hypothesis, this method of data collection produced more precise results with narrow confidence intervals. The confidence intervals also had a greater range below zero, showing a greater likelihood that the true mean would show a reduction at the 12-month time point. Additionally, as the sample size is small, even a slight increase in a participant's breast elasticity could influence the results and lead to statistically insignificant results. With this sample size, the confidence intervals may be a better indication of efficacy in this scenario. Further explanations for the insignificant results could be that as the mammograms were only conducted at baseline and month 12, it is unable to determine if the changes within the %VBD and TFV also plateaued at the three-month mark, so it cannot be concluded that the elasticity changes reflected changes in the mammography variables. Based on the confidence intervals, it can be hypothesised that the elasticity does decrease across the 12 months, but the sample size may have influenced the findings. Overall, as seen in the HAVAH*T+Ai*™ study, reductions may occur in the breast elasticity in response to the interventions. Future research needs to be done with more regular outcome measures for both breast elasticity and mammography for %VBD and TFV.

The next analysis was the correlations between the mammography variables and breast elasticity, and the differences in these correlations when using 6 x 3mm Q-Box™ and the Free-Trace Q-Box™. From the results of this study, descriptively, there were several variations between the different Q-Box™ protocols. An example of this was from the enobosarm and anastrozole study, analysing the correlation between the change in %VBD and the change in tissue elasticity across 12-months. The original Pearson's *r*-value was 0.315, and the subsequent *r*-value was -0.12, although both were statistically insignificant, the change in the method of data collection has changed the direction of the correlation, which has the potential to lead to a dramatically different conclusion being made from the data. However, as most of the correlations were not statistically significant, these changes could have occurred due to chance and may not be replicated with a larger study.

From these new data analyses conducted in this chapter, it was re-evaluated whether the original conclusions in regard to the thesis objectives were still correct or whether these have

changed, and if so, what are the new conclusions that can be drawn from the data. The original objectives of the HAVAH*T+Ai*™ study were to determine the potential effects of HAVAH*T+Ai*™ on breast tissue elasticity, as measured by SWE and whether these changes correlate with changes in MBD. The initial results, using the data collected with the 6 x 3mm Q-Box™, demonstrated that there were statistically significant changes from the second month of the study, and this continued for the third month with again statistically significant breast elasticity decreases. From the baseline values to month two, the 95% CI showed values of -6.72 to -0.87 with a p-value of 0.004, and the baseline to month three results produced a 95% CI of -7.31 to -2.78 with a p-value of less than 0.0005. These low p-values and CI that favour more significant decreases, alluded to a lower risk of the results occurring due to chance and demonstrated quite significant effect sizes in response to the intervention. The analysis of this study using the Free-Trace Q-Box™ elasticity data, showed very similar results with a significant median decrease of -3.70kPa at two months with a p-value of 0.002 and the median decrease of -4.35kPa at three months with a p-value of less than 0.0005. These results also present very low p-values which demonstrates a low risk of chance affecting the results. The similarities between the results show that even when using a technique that produces more precise data, the same conclusions can be drawn from the data, this being that breast elasticity may be used as a biomarker of early response to changes in the breast tissue and further research is warranted.

The secondary objective of this study was analysing the correlations between the mammography variables and the elasticity values. As previously discussed in Chapter 4, the most important correlation that needed to be evaluated was the change in the elasticity data and the change in TFV. In regard to the HAVAH*T+Ai*™ study, when observing the change in TFV at 12 months and the change in breast elasticity at three months with Free-Trace Q-Box™ data, there were both statistically significant moderate correlation between the two variables. In addition, when looking at the correlations, with elasticity at one month and TFV at 12 months, both analyses showed statistically significant moderate correlations. These results demonstrated that within the HAVAH*T+Ai*™ study, there might be a relationship between the changes in breast elasticity and TFV, which could be confirmed with more research.

In regard to the anastrozole and enobosarm study and the change in breast elasticity and TFV, the Free-Trace Q-Box™ data produced a moderate, positive relationship which was approaching the traditional level of statistical significance. When looking at the elasticity change at one month to the TFV across 12 months, there was an r-value of 0.459 (p=0.253), which was close to the original calculation with a moderate correlation of 0.500 (p=0.313). Both of the results (old and new) demonstrate that elasticity changes may correlate with TFV changes, and elasticity may have the potential to be able to predict an early response of the changes in TFV across 12 months of chemopreventative use.

These new results are still favourable for the hypothesis that the changes in elasticity correlate with the changes in the mammography variables. However, this hypothesis cannot be ruled out as the sample size of the study is small and small variations in the individual's participants results can dramatically change the outcome of the study. A larger study needs to be conducted to determine if the breast elasticity changes at one month can correlation with the TFV changes.

7.6 Conclusion

The findings within this chapter demonstrate that even when using a more precise method to calculate the breast elasticity, there were still statistically significant reductions in the breast elasticity in response to both interventions. These results were seen at the three-month time point in both the studies and with statistically insignificant reductions at the 12-month time point in the enobosarm and anastrozole study. These results also show that there are still moderate correlations between changes in breast elasticity and changes in TFV and these correlations may also be seen as early as one month in the breast elasticity measures.

Chapter 8 The Behaviour of Breast Elasticity as Measured by Shear Wave Elastography in Healthy Women in Regard to Menstrual Cycle Changes, Repeatability and Intra-rater Reliability.

8.1 Background

Within the first two experimental studies, as previously commented on, it was observed that there was substantial within-subject variation with repeated measures when using the SWE to measure whole breast elasticity. This observation showed that with some of the repeat measures, the breast elasticity would decrease and then subsequently increase with the following measurement. In the absence of a control group, there is uncertainty as to how much of this variability are genuine changes in the breast and related to hormonal fluctuations or other factors, and how much is due to measurement error. This study was designed to provide information on these unknowns, analysing the behaviour of breast elasticity through repeat measures in women without breast disease and who are not using hormonal interventions of any kind. This will provide more insight into the results from the other trials within this thesis.

In addition, as using SWE to measure the whole breast elasticity is a new indication and is not widely researched, this study aimed to calculate the intra-rater reliability of SWE on whole breast elasticity. This analysis allowed us to determine if any of the measured changes are occurring due to the interventions in the HAVAH*T+Ai*™ study and the enobosarm and anastrozole study, rather than the assessor's technique or issues with the images acquired.

This study is a reliability and repeatability study with four repeat measures, every two weeks on 19 women who were free from breast disease and any form of systemic hormonal intervention. Whole breast elasticity and stage of the menstrual cycle were assessed and recorded for each participant. The images were also obtained using two patient positions to measure the effect of different patient position on the whole breast elasticity.

8.2 Objectives

The primary objectives of this trial were to determine the behaviour of breast tissue elasticity with repeat measures on healthy women and to determine whether the hormonal fluctuations that occur with the menstrual cycle can influence the breast elasticity measurements.

The secondary objectives of this trial were to:

1. Determine the intra-rater reliability of using SWE to measure the whole breast elasticity in a healthy cohort.
2. Determine whether the arm position changes the breast elasticity output.
3. Determine the normative breast elasticity values of this healthy cohort to use as a comparison for other studies within this thesis.

8.3 Publication Manuscript

The Statement of Authorship for this publication manuscript is presented in Appendix 5.

The Behaviour of Breast Elasticity as Measured by Shear Wave Elastography in Healthy Women in Regard to Menstrual Cycle Changes, Repeatability and Intra-rater Reliability.

1st author: Daniella Dougherty, The University of Adelaide

2nd author: Dr Stephen Birrell, HAVAH Therapeutics Pty Ltd; Wellend Health Pty Ltd

3rd author: Professor Paul Rolan, The University of Adelaide

Corresponding Author:

Daniella Dougherty

Email: Daniella.dougherty@adelaide.edu.au

Mobile (+61) 405 483 861

Abstract

Introduction

Shear wave elastography (SWE), is an imaging technique that analyses the elasticity of a tissue or organ of interest. SWE has proven to be valuable in breast assessments, and there are emerging indications for using the assessment of whole breast elasticity. This study aims to determine the behaviour, influence of hormonal fluctuations, patient position and intra-rater reliability on breast elasticity across four repeat measures.

Method

19 premenopausal women who were absent of any breast disease, and not on any form of hormonal interventions had four repeat SWE measurements, two weeks apart in two different patient positions. Each patient had their stage of menstrual cycle determined via blood analysis. Statistical analysis was conducted to determine the intra-rater reliability, the difference in breast elasticity across the different stages of the menstrual cycle and difference in elasticity measures using differing patient position. The mean whole breast elasticity was also calculated.

Results

Mean breast elasticity during the follicular stage was 6.61kPa (SD 1.86) and 6.70kPa (SD 2.01) during the luteal stage; this was a statistically insignificant difference (p= 0.670). The breast

elasticity was not statistically different at any time point (p=0.602). There was a statistically significant median difference of -1.16kPa (p<0.005) between the two patient positions. The intra-rater ICC was 0.98 (95% CI 0.97 to 0.99; p<0.005) for the whole breast measurements.

Conclusion

Average whole breast elasticity is a reproducible measure for healthy women and has the potential to be used as an outcome measure to determine the efficacy of therapeutic interventions for breast conditions.

Introduction

Shear wave elastography (SWE) is an elastography technique that analyses the soft tissue mechanical properties of the tissue or organ of interest. SWE uses acoustic radiation force impulses, which provide the mechanical excitation through pushing beams that deform the underlying tissue of interest. Several of these pushing beams are transmitted at different depths, which results in the propagation of transient shear waves. The speed of these shear waves is then measured using a scanner with a very fast frame rate, allowing the shear waves to be followed in real-time. This is repeated for different lines; allowing a map of a region of interest (ROI) to be created from analysing the differences in arrival times and calculating the shear wave speeds. A colour-coded image is then displayed on the SWE monitor, and the quantitative data is presented as a measure of shear wave speed in meters per second ($m/s\text{-}^{1}$) or converted to the Youngs Modulus and displayed as kPa. Throughout the measurement, B-mode image guidance is possible as the same transducer that generates the shear wave also captures their propagation (Bercoff, Pernot et al. 2004, Bercoff, Tanter et al. 2004, Sebag, Vaillant-Lombard et al. 2010, Shiina, Nightingale et al. 2015).

To date, within the realm of breast health, the main use of SWE has been differentiating breast lesions as being either benign or malignant, with current research showing that there is a statistically significant difference in elasticity values between benign and malignant lesions, in which malignant lesions predominately have a greater elasticity (Athanasiou, Tardivon et al. 2010, Chang, Moon et al. 2011, Berg, Cosgrove et al. 2012, Au, Ghai et al. 2014). For this indication, the operator places a Q-Box™ (a tool used to calculate the area of elasticity) on the SWE image within the area of the greatest elasticity in the lesion to generate the maximum or

mean elasticity of the lesion. This indication has been proven reliable with intra-observer reliability for maximum and mean elasticity reported as high (ICC = 0.84 and 0.87) and the interobserver agreement for maximum elasticity quantification having a Cohens Kappa value of 0.66 (95% CI 0.59 to 0.73) (Cosgrove, Berg et al. 2012), which represents a substantial level of agreement between the two observers (McHugh 2012).

SWE has proven to be extremely valuable in breast assessments, and with this there are new emerging indications that are aiming to assess the whole breast elasticity; rather than the selective area of interest. These indications include correlation of the breast elasticity with the BI-RAD breast density assessment (Evans 2015). For this indication, the elasticity was obtained using a ROI as large as possible within the SWE image rather than selectively choosing an area of tissue. There has also been research into monitoring breast pain, inflammation and capsular contracture (Rzymski, Kubasik et al. 2011, Rzymski, Kubasik et al. 2011, Sowa, Yokota et al. 2017). These new indications use different methodologies to that listed above, as rather than finding the area of greatest elasticity, the whole breast elasticity is sought. For this, the researcher needs to take an image in each quadrant of the breast and select a ROI to calculate the elasticity of each quadrant and then the breast as a whole. This type of imaging may be used to detect changes within the breast tissue in response to therapeutic interventions. Therefore, it is important to know the typical behaviour of breast elasticity in a healthy cohort upon repeat measures, this including the effect of natural hormonal fluctuations with the menstrual cycle, examination the reliability of using SWE, and determination if the patient position has a significant effect on the elasticity measurement. By gaining this information, researchers and clinician will have more knowledge about breast elasticity and feel more confident using it as an outcome measure in clinical practice or future health research.

Therefore, the aim of this study was to determine the behaviour of breast elasticity across four repeat measures, analyse the intra-rater reliability of whole breast elasticity when using SWE, and determine if the patient position can significantly alter the elasticity output in a healthy patient cohort.

Material and Methods

This study was approved by the Institutional Human Research Ethics Committee at The University of Adelaide (approval number H-2018-197.) Informed consent was obtained from all participants. Subjects were recruited from the Burnside Breast Centre and Wellend Health Pty Ltd, located in Toorak Gardens, South Australia, through Facebook advertising, and through word of mouth of the participants. The study population consisted of premenopausal women between the ages of 18 and 50 years, who were currently not pregnant or lactating. Participants were excluded from this study if they had the presence of breast cancer, had breast implants, had any previous breast surgery, the presence of a pacemaker or are currently on any hormonal treatments for mammographic breast density (MBD) or systemic contraceptive medications with the exception of the Mirena. The participants were required to attend four visits, one every two weeks, each comprising of a blood test to measure progesterone to determine the stage of the menstrual cycle and the SWE imaging.

Determination of Stage of the Menstrual Cycle

Forearm venous blood samples (5ml) were collected for each participant prior to the SWE imaging at each visit. The blood was allowed to clot, and the serum was separated by centrifugation, collected and stored at -80° until further use. Assays were performed by a commercial pathology laboratory. Paramagnetic particle-based enzyme immunoassay was used for the determination of progesterone from serum samples. The reference limits for the follicular stage were <3nmol/L, and the luteal phase was 5-75nmol/L.

Shear Wave Ultrasound Protocol

The elasticity was evaluated using the Aixplorer® ShearWave™ Ultrasound machine (Supersonic Imagine, Aix-en-Provence, France) with a SuperLinear™ SL15-4 linear transducer with a bandwidth of 2-10 megahertz, in the default breast pre-set, running in SWE mode. Two different positions were utilized during this study, firstly the volunteer lying supine with arms relaxed by their sides (position A), the second position was the volunteers lying supine with the ipsilateral arm to the breast being imaged resting under their head (position B). A single operator conducted all the scans for all the participants and all the repeated measures. The imaging protocol is presented in (Figure 8-1), the breast was divided into four quadrants, the

outer lower, the outer upper, the inner upper and the inner lower. Each quadrant was scanned from the left to right breast, three times in position A and once in position B. The transducer head was positioned approximately 2cm away from the nipple and was placed on the skin in a parallel manner from the nipple (Figure 8-2).

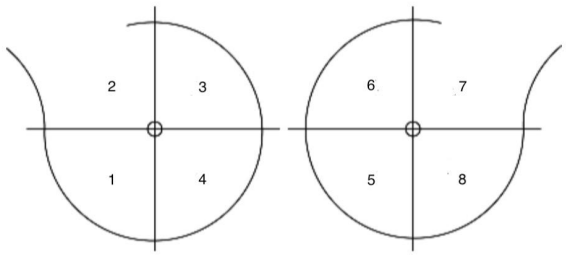

Figure 8-1: Image sequence of the breast quadrants used in shear wave elastography

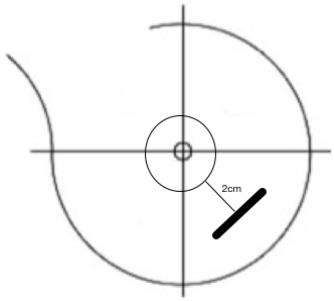

Figure 8-2: Positioning of the transducer head on the breast

Once the images were acquired, the elasticity values were generated by using the Q-Box™ trace function to trace the largest ROI from the image omitting any areas for which the shear waves had not propagated (black holes) or any areas for which ultrasound artefacts were seen on the image (as these lead to excessively high elasticity values that do not correspond to figures on the B-Mode ultrasound image) (Figure 8-3), this again generated values for the E_{min}, E_{mean}, E_{max} and SD for each quadrant. The E_{mean} of each quadrant were used to produce the mean elasticity for each breast and both left and right breasts combined.

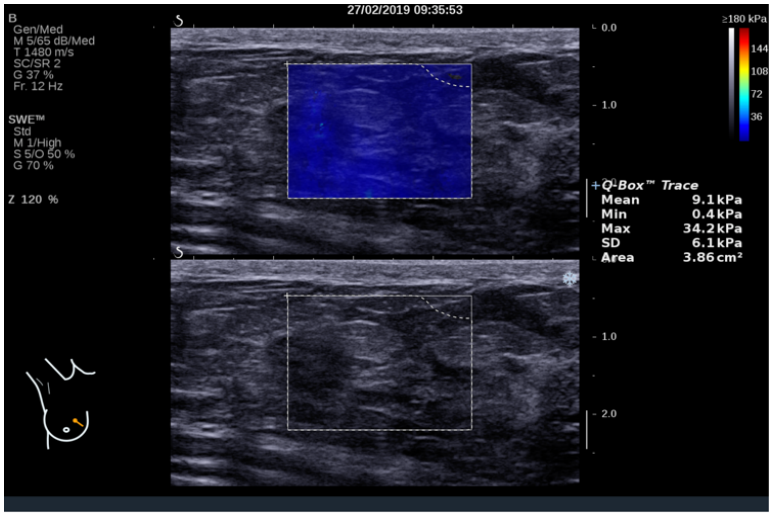

Figure 8-3: Shear wave ultrasound image with Free-Trace Q-Box™, avoiding a black hole

Statistical Analysis

All data were entered into in Microsoft Excel (Microsoft, USA), and all statistical analyses were performed using SPSS Version 25 Software (IBM, Armonk, NY, USA). The paired samples T-test for parametric data, and the Sign test for non-parametric data were used to determine if there was a difference in the within-subject breast elasticity values during the different periods of the menstrual cycle, and whether there was a difference in the breast elasticity when the participant had their arms relaxed by their side or resting above their head. For these analyses, data was presented as median or mean (SD) unless otherwise stated. The interquartile range (IQR) was used to determine which patient position had the least amount of dispersion within the data. The Friedman test was used to analyse the behaviour of the average breast elasticity with the repeated measures. Intra-class correlation coefficient (ICC) analysis was used to determine the intra-rater reliability of the measurements. Data were considered statistically significant at $p<0.05$ and if the confidence interval (CI) did not cross zero.

Results

19 women volunteered to be included in the study; the mean age was 30.56 years (SD 9.10) with a mean BMI of 25.04. The patient characteristics are presented in Table 8-1. The average estimates of the Young's Modulus for both breasts combined were 6.80kPa (SD 2.13kPa).

Table 8-2 shows the average breast elasticity of each quadrant for each repeated measure and the change in position for the participants.

Table 8-1: Participant Characteristics

Demographic	**Average (min-max)**
Age	30.56 years (23.25 – 48.77)
Weight	68.88kg (50 – 90)
Height	166.25cm (155 – 176)
BMI	25.04 (19 – 32)
% of nulliparous participants	78.95%
% of participants using Mirena	36.84%
% of participants who previously used systemic contraception	73.68%
Length of previous systemic contraception use	3.12 years (0.66 – 14 years)
Race	
Caucasian	100%
Asian	0%
African	0%
Other	0%

Breast Elasticity and Stage of Menstrual Cycle

The paired-samples t-test was used to analyse the difference between the average breast elasticity in the luteal and follicular stage of the menstrual cycle. With the arms relaxed the mean elasticity during the follicular stage was 6.61kPa (SD 1.86) and during the luteal phase 6.70kPa (SD 2.01). There was a mean difference of 0.09kPa (95% CI -0.37 to 0.55; p=0.670) between the two menstrual cycle stages. This was a statistically insignificant difference in the elasticity values in the follicular or luteal stage of the menstrual cycle. In regard to the position of the arms resting above the participants head, the mean elasticity during the follicular stage was 8.26kPa (SD 2.98), and the luteal phase was 8.38kPa (SD 3.13). This was a statistically insignificant difference of 0.12kPa (95% CI -0.54 to 0.30; p=0.559).

Breast Elasticity with Repeat Measures

The Friedman test was used to analyse the average breast elasticity over four repeat measures. The average elasticity for each time point is reported in Table 2. The breast elasticity was not statistically different at any time point (p=0.602).

Table 8-2: Median whole breast elasticity at each time point

Measure	Elasticity kPa (SD)
1	6.53 (1.93)
2	6.75 (1.84)
3	6.99 (2.59)
4	6.77 (2.15)

Breast Elasticity and Participant Position

The descriptive statistics for this data showed the mean elasticity for the arms relaxed was 6.76 kPa (SD 2.12), and the arms above the participant's head was 8.31 kPa (SD 3.10) with a mean difference of 1.55 kPa, all of the results are reported in Table 3. As the data was non-parametric, the Sign test was used for the analysis. The results from the Sign test show that the median elasticity for arms relaxed was 6.19 kPa, and the arms above the participant's head was 7.53 kPa; this was a statistically significant median difference of -1.16 kPa ($p<0.005$). The IQR was compared for each of these positions, the IQR for the arms relaxed data set was 3.38, and the IQR for the participant's arms resting above their head was 4.75.

Table 8-3: Mean breast elasticity (SD) for each quadrant in position A and B

	Position A				Position B			
	Measure 1	**Measure 2**	**Measure 3**	**Measure 4**	**Measure 1**	**Measure 2**	**Measure 3**	**Measure 4**
Lower Outer	6.20 (2.02)	6.38 (2.41)	6.47 (2.97)	6.30 (2.43)	7.65 (3.42)	8.01 (3.39)	8.06 (3.82)	7.78 (3.07)
Upper Outer	6.86 (3.00)	7.40 (2.55)	7.54 (3.65)	6.83 (2.67)	6.90 (3.26)	7.58 (3.08)	8.06 (3.84)	7.64 (3.08)
Upper Inner	6.73 (2.11)	7.00 (2.43)	7.26 (2.84)	7.51 (2.58)	8.18 (3.01)	8.90 (3.99)	10.12 (4.72)	10.15 (4.54)
Lower Inner	6.34 (1.69)	6.22 (1.69)	6.67 (1.91)	6.46 (2.14)	7.93 (2.39)	8.48 (3.24)	8.74 (2.98)	8.71 (3.70)

Intra-rater Reliability

There were 76 datasets (4 quadrants for each breast, each with three repeat measurements), the ICC was used to determine the reliability. The reliability of the SWE measurements made by the same operator was excellent with an ICC of 0.98 (95% CI 0.97 to 0.99; $p < 0.005$).

Discussion

SWE is a relatively new form of radiographic imaging that is gaining more traction in regard to breast health. Previously in regard to the breast, it has been used to differentiate between benign or malignant lesions. However, more indications for this imaging modality are beginning to be researched and utilised in clinical practice. For this reason, it is imperative to know the behaviour of breast elasticity in women without any breast disease. The population of disease-free women in this cohort had an average breast elasticity of 6.80kPa, which was in line with a previous study on healthy subjects by Li, Wang et al. (2015) who reported a mean elasticity for glandular tissue of 6.60kPa and fatty tissue of 4.86kPa. These results were lower than Rzymski, Skórzewska et al. (2011) who found that glandular tissue had a mean elasticity of 11.28kPa and fatty tissue of 9.24kPa. Both of these studies reported the glandular and fatty tissue separately, which explains the slight variation within the results. The only study using the full trace function, similarly reporting the average elasticity, was Evans (2015) who reported an average elasticity of 10-13kPa in women; however, these women had varying levels of breast density ranging from A to D on the Bi-RAD scale, this meaning the data was not normative as dense breast can be considered pathological.

The main focus of this study was to determine the behaviour of breast elasticity on repeat measurements and whether the stage of the menstrual cycle influenced these results. Female sex hormones, especially oestrogen and progesterone, influence the structure and composition of multiple tissues inclusive of the breast parenchyma (Lorenzen, Sinkus et al. 2003, Rzymski, Wilczak et al. 2012, Kaaks, Tikk et al. 2014). Oestrogens are well-known stimulators of collagen biosynthesis and cell growth in several cell types (Beldekas, Gerstenfeld et al. 1982, Ernst, Schmid et al. 1988, Surazynski, Jarzabek et al. 2003). Cell proliferation is lowest during the follicular stage of the menstrual cycle because of low oestrogen and progesterone levels. During the luteal stage, there are higher levels of

oestrogen and progesterone, which leads to significant changes in the breast lobules and the proliferation of the breast epithelium reaches its peak (Pike, Spicer et al. 1993, Dimitrakakis and Bondy 2009). Additionally, Hussain, Roberts et al. (1999) documented a correlation between the plasma levels of progesterone and changes in breast volume. Consequently, the influence of oestrogen and progesterone on cell proliferation and collagen synthesis may influence the breast elasticity. This study reported that there was only a small difference between the two stages of the menstrual cycle, with the luteal stage having a slightly higher elasticity. This difference was not statistically significant. These results were similar to Li, Wang et al. (2015) which found no significant differences in elasticity between glandular and adipose tissue throughout the menstrual cycle, with the glandular tissue elasticity being lower on the luteal phase than in the early follicular phase. These results demonstrate that the hormone fluctuations that occur throughout the menstrual cycle do not significantly influence the breast elasticity. If being used for longitudinal studies or for clinical assessment, these results show that the stage of the menstrual cycle does not need to be taken into account for when participants are to be imaged, and any changes that occur within the elasticity may be due to other factors.

Additionally, within longitudinal studies or clinical practice, to determine if a therapeutic intervention were able to influence the average breast elasticity, it is necessary to know that the elasticity remains stable in disease-free women who are not on any form of intervention and that the elasticity would not be influenced by the operator. Within this study, it was found that in this demographic, the average breast elasticity did not change over four repeat measurements. The results also showed that there was excellent intra-rater reliability with the SWE measurements. These findings demonstrate that if used in a longitudinal study, any changes that were seen within an intervention group are more likely due to the intervention and not measurement errors.

The final variable of this study analysed was whether a change in position would affect the average breast elasticity. As SWE for the breast is a relatively new imaging modality, there is no protocol for the best position to conduct the imaging. This study demonstrated that there were statistically significant differences in the breast elasticity from when the participant had their arms relaxed by their side or resting above their head. This indicates that there needs to

be consistency within the participant position for longitudinal studies; otherwise, the results could be affected. This information may also be important when comparing the results of different studies. Due to the decreased IQR, the authors of this study recommended using arms relaxed by the participants side.

Conclusion

These findings suggest that the stage of the menstrual cycle does not influence the average breast elasticity. In addition, within a disease-free population, the breast elasticity does not change over four repeat measures and the intra-rater reliability is excellent. Consequently, average breast elasticity is a reproducible measure and may be used as an outcome measure to determine the efficacy for therapeutic intervention for breast conditions.

Conflicts of Interest

There were no conflicts of interests to declare

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8.4 Publication Summary

This study was conducted due to the variation seen within the other studies utilising SWE to measure breast elasticity. It needed to be researched as to whether these fluctuations that were seen in the breast elasticity were due to the effect of the hormonal combinations or were due to other influencing factors such as operator changes or error, stage of the menstrual cycle or the overall reliability of the SWE machine. This study recruited 19 premenopausal women between the ages of 18 and 50 years, with an average age of 30.56 years who were currently not pregnant or lactating or on any form of hormonal intervention with the exception of the Mirena.

The first interesting finding from this study was the average breast elasticity was 6.80kPa (± 2.13kPa), this is significantly different from the other two experimental studies that utilised the SWE with the baseline measurement for those studies being 15.18kPa for the study utilising enobosarm and anastrozole and 12.81kPa for the study utilising HAVAH*T+Ai*™. When the data from these two studies were combined the average elasticity was 13.81kPa (±3.94), which when using the Welch test demonstrated a statistically significant mean difference of 7.28kPa (95% CI 5.21 to 9.34; $p<0.0005$) between the data produced in this study. The MBD of the participants within this study was not measured, however taking a random sample of women with no breast pain is more likely to represent a group with a lower %VBD than a group purposively recruited for having a measured high MBD. For this reason, it can be hypothesised that women with a greater MBD may have a greater elasticity when compared to a healthy cohort. This is a promising finding for the overall aim of this thesis to determine if elasticity, as measured by SWE, is a viable biomarker for MBD as with future research it could be determined whether the baseline correlation of MBD correlated with breast kPa.

The second important finding of this study is that within this cohort, the stage of the menstrual cycle did not influence the breast elasticity. There was a statistically insignificant difference of 0.09kPa (95% CI -0.37 to 0.55; $p=0.670$) when comparing the follicular to the luteal stage of the menstrual cycle. This is a beneficial finding as this shows that the timing of the SWE in relation to the menstrual cycle should not dramatically influence the output. The third important finding was that there was no statistically significant difference in kPa at any

time point with the repeat measurements (p=0.602). Both of these findings are of importance, as the consistency within this study can allude to the changes being seen in the previous studies being due to the hormonal intervention rather than other influencing factors, such as the menstrual cycle hormone fluctuations on the repeatability of the SWE machine. This also has more supporting evidence as the intra-rater reliability was excellent with an intra-class correlation coefficient of 0.98 (95% CI 0.97 to 0.99; p<0.005).

The final important finding of this study was the statistically significant differences between the elasticity outcomes of patients who had their arms relaxed compared to their arms above their head. The median elasticity for the arms relaxed position was 6.19 kPa, and for the arms above head position was 7.53 kPa, this was a median difference of 1.16 kPa. These results demonstrate that changing the position of the participants' arms can significantly change the results. The implications of this finding show that the participant position needs to be kept constant with repeat measures as it could lead to clinically significant differences. The researcher needs to monitor this, as frequently during the SWE examinations during this study and the other studies within this thesis, participants would change their arm position for comfort reasons and required prompting to return their arms to the position required. If this is not kept constant within measures or with repeat measures, there could be significant changes occurring which are solely due to the arm position.

8.5 Conclusion

Overall, this study demonstrates that SWE when being used to measure the average breast elasticity is a repeatable and reliable method and is not greatly influenced by fluctuating menstrual hormones or the operator, the only factor discovered within this study that can influence the average breast elasticity is the patient position. Although further research is required, when using SWE to determine the pharmacodynamic effect of a hormonal intervention, there is promising evidence suggestive that the changes occurring are due to the intervention.

8.6 Future Research

Future research will need to be conducted with women who have high MBD not on hormonal intervention. This will help determine if these women have different reliability psychometric properties or breast behaviour when their breast elasticity is being measured by SWE.

Chapter 9 Discussion and Conclusion

9.1 Overview

Breast cancer is one of the most prevalent types of cancer among women, and it is a sizeable universal medical issue. Mammographic breast density is a major independent modifiable risk factor for breast cancer, and it has been predicted that a third of breast cancer could be linked to a woman having highly dense breasts (Boyd, Martin et al. 2010). Currently, MBD is being considered as an appropriate baseline breast cancer risk marker, and a surrogate endpoint for assessing therapeutic interventions; this due to the strong association between MBD and risk of breast cancer. These considerations also allow MBD to be used as a biomarker to evaluate the efficacy of interventions that are aimed at reducing breast cancer risk.

Within the first chapter of this thesis, a literature review was conducted regarding the use of mammography and MBD as a surrogate endpoint or biomarker for clinical trials and several limitations were identified. These limitations were listed in Section 1.4.1 and include the subjective nature of the interpretation of MBD when using a non-automated method of MBD quantification. Non-automated methods can also lack the sensitivity to see a significant change in the MBD values, which can lead to Type 2 errors and insignificant results in clinical trials. Furthermore, the increased imaging quality that comes with improved technologies can lead to improved results when using automated methods. In addition, different mammography machines can lead to significantly different MBD values for the same patient (Kerlikowske, Ichikawa et al. 2007, Vachon, Pankratz et al. 2007, Lokate, Stellato et al. 2013, Work, Reimers et al. 2014). Breast pain is also quite common in women, and in particular in women, with high MBD; this breast pain and fear of breast pain is a prominent reason for women choosing not to attend mammography visits and also for not returning for follow up mammograms if they did attend one visit (Kee, Telford et al. 1992, Marshall 1994, Straughan and Seow 1995, Elwood, McNoe et al. 1998). The reported fear of breast pain may lead to recruitment issues or high attrition rates in research. Another limitation of mammography is the ionising radiation that is delivered with each image, which can accumulate during longitudinal studies where multiple imaging sessions are required, which in itself is a breast cancer risk.

One of the most significant limitations of using MBD variables as a biomarker during clinical trials is the relative time-insensitivity to detect changes in the mammography variables (MBD, %VBD, PMD, TFV, TBV). Despite a drug having an immediate pharmacological effect, any potential resultant improvement in breast structure may not be seen for a year, this being either due to the typical interval used for mammographic imaging or the time required for the intervention to produce an effect on the tissue structure of the breast. Hence long clinical trials may need to be designed and implemented to determine whether a treatment is effective. Concerning this, within a clinical environment, women on anti-oestrogen therapies (which usually have considerable adverse effects) do not have a method of providing timely feedback of their response to their intervention, which may contribute to the low compliance rates seen with some of these treatments. Due to these limitations, it was concluded that there was a knowledge gap regarding a biomarker for MBD for the early detection of changes in the breast tissue. Ideally, if a biomarker was found, it could be used in both clinical research and within clinical care settings.

Breast elasticity appeared to be a potentially viable option as a biomarker of MBD. The reasons for this being that high MBD tissue has extensive collagen, a more significant number of cells, increased ECM and a low adipocyte component (Alowami, Troup et al. 2003, Li, Sun et al. 2005, DeFilippis, Chang et al. 2012). These histological components can lead to tissue stiffening, as is seen in malignant breast lesions, and this would lead to increased measurable elasticity of the tissue of interest.

This thesis investigated whether whole breast elasticity, as measured by SWE, can be used as a biomarker for MBD and to establish a reliable and valid protocol of using SWE to measure whole breast elasticity. This chapter will review how the studies included in this thesis contributed to the research and development of whole breast elasticity as a biomarker for MBD. This chapter will be separated into the following sections;

1. The use of breast elasticity as a biomarker for MBD
2. The protocol that should be used to determine whole breast elasticity
3. The limitations of this research
4. The clinical and research implications of this work
5. Conclusion

9.2 The Use of Breast Elasticity as a Biomarker for Mammographic Breast Density

The first overarching aim of this research program was to generate evidence into the validity of using whole breast elasticity as a biomarker for MBD. Four different studies were conducted regarding this research aim. The first key point was that when using SWE to measure whole breast elasticity, there was a very strong intra-rater reliability with an ICC of 0.98 (95% CI 0.97 to 0.99; $p<0.0005$). Having a strong intra-rater reliability score is essential in longitudinal studies; strong reliability allows us to increase our confidence that repeated SWE measures will remain consistent when measured by a single individual if no changes are occurring within the breast tissue (Roach 2006). Another important psychometric property is the inter-rater reliability; however, for feasibility reasons during this research program, this measure could not be calculated and will need to be examined with further research. In addition to this, within a population of women not on any hormonal intervention, with four repeat measures, breast elasticity was not statistically different at any time point. From these results, we can conclude that foremost, breast elasticity measured using SWE with a single operator is a reliable outcome measure and has favourable psychometric properties that would make it a desirable outcome measure for use in clinical trials. However, as will all research, these studies will need to be replicated or expanded to further validate the findings presented.

The second key point to examine is the baseline normative breast elasticity values. Two of the studies which utilised SWE had recruited women with high MBD (objectively measured with VolparaDensity™) and one study recruited women from the general population who had not previously had a breast density assessment. Within this third study, it could be hypothesised that the population had a lower mean breast density, this being due to the prevalence of high MBD in the Australian population (with approximately 50% of the population potentially being categorised as heterogeneously dense breasts or extremely dense breasts) (Cording, Smith et al. 2018). The participants in this group also did not have breast pain, which is commonly associated with high MBD. The average elasticity of the women within the third study who were hypothesised as having lower MBD was 6.80kPa (± 2.13kPa) and the mean breast elasticity from the studies with women with quantified high MBD was 13.81kPa (±3.94kPa). This resulted in a statistically significant difference of 7.28kPa. On face value, this reveals that

women recruited due to a higher MBD had greater elasticity than a sample of the general local population. As the MBD was not formally measured in the healthy population group, future studies need to be conducted in a cross-sectional manner to determine whether there is a relationship between MBD and breast elasticity, recruiting a broad sample of women, from varying age groups, with varying MBD values. Future research could also aim to correlate the baseline TFV and breast elasticity, as TFV represents the volume of the fibrodense region of the breast. Through this research program, early evidence has demonstrated a potential trend for women with high MBD to have a greater baseline breast elasticity, which contributes to the evidence supporting breast elasticity being a biomarker for MBD, warranting further investigation.

The next key finding was that breast elasticity responds and decreases (in a rapid manner) to hormonal interventions that have also been shown to reduce %VBD and TFV. The initial study in this thesis (Chapter 3) was a cohort analysis of a patient database of women who were administered HAVAH*T+Ai*™. This study concluded that there was a correlation with the use of HAVAH*T+Ai*™ and the reduction of %VBD and TFV. The initial experimental trial (Chapter 4: The HAVAH*T+Ai*™ study), was a three-month pharmacodynamic sub-study within a pharmacokinetic study of HAVAH*T+Ai*™. This study revealed that breast elasticity decreased with a single dose of HAVAH*T+Ai*™; the mean elasticity decreased consistently from baseline to the three-month time point, reaching statistical significance at the second and third month. In addition to the overall breast elasticity decreases, the final mean elasticity was 8.77 kPa (±3.67kPa), which is approaching the hypothesised normative values previously reported of 6.80kPa in Chapter 8.

Generally, a similar pattern was observed within the enobosarm and anastrozole study (Chapter 5); however, this cohort of women started with a higher mean elasticity at baseline, and even with the decreases throughout the study, the values did not approach the hypothesised normative values of 6.80kPa. These decreases were statistically significant at the three-month time point, with a reduction also being recorded at one month. As this trial had an additional breast elasticity measure at month 12, this time point demonstrated a slight statistically insignificant increase in elasticity compared to the three-month time point. It can be observed that both of these studies had a statistically significant elasticity decrease in the first three months from the commencement of the intervention. However, what is unexplored

is the histological changes occurring with the breast tissue after this time point. When using the 6 x 3mm Q-Box™, the results demonstrated a statistically significant reduction at the 12-month time point. This reduction is what we hypothesised would represent the changes occurring in the breast tissue and what we believed to be the true results. However, these results are not consistent with the Free-Trace Q-Box™ results, and there are a few reasons why this could have been the case. Firstly, these results may be the correct findings as no previous research has been conducted in this area; although it was hypothesised that further reductions should be seen at the 12-month time point, the reductions may plateau after the three-month time point. Secondly, the small sample size of the study lead to small fluctuations within the individual participant results, which had a large impact on the mean overall values. For these reasons, it would be beneficial to conduct further research, with a formal sample size calculation, to produce significantly powered results. The data generated in this thesis would enable such a calculation. Future studies could also be designed with a more frequent outcome measure assessment schedule; this could include both SWE and mammography imaging. As we hypothesised that the changes in breast elasticity might occur before the changes in the breast tissue that lead to quantifiable changes in %VBD and TFV, it would be beneficial to create a time profile of the elasticity and MBD changes occurring. Knowing this information would also aid the design for future research, in particular, concerning the design of the schedule of assessments.

A further key finding was the trend of strong correlations between the changes in breast elasticity and changes in TFV, and the early time point for which these changes correlated. In addition to this was the lack of a correlation between the changes in elasticity and the changes in %VBD. The HAVAH*T+Ai*™ study demonstrated a trend of negligible correlations between the changes in %VBD from the baseline measure to the month 12 measure and the breast elasticity changes from baseline to month three. The enobosarm and anastrozole study had similar results with the correlation analyses between the change in breast elasticity and change in %VBD from baseline to month 12 showing a trend towards a low correlation. These findings demonstrate that within both of these studies, there was a trend towards a negligible and low correlation between the changes in %VBD and the changes in elasticity. These results establish that within our study populations, there was not a relationship between these two variables. However, this low correlation was hypothesised, as with both the HAVAH*T+Ai*™ and

enobosarm and anastrozole interventions, the TFV and TBV decreased. The resultant effect of this is %VBD not reducing to the same degree as the other two variables, or it may even remain stable as the percentage is calculated from these two variables.

Due to the reasons mentioned above, the change in TFV and breast elasticity was considered the more relevant correlations to explore. This is because the change in TFV reflect changes in the absolute fibrodense areas of the breast, which is the area hypothesised to be positively associated with breast cancer risk as this is the actual tissue where breast cancer develops (Haars, van Noord et al. 2005, Ursin, Hovanessian-Larsen et al. 2005). Furthermore, breast elasticity changes occur in response to early functional changes within the structure of the breast tissue, whereas measurable changes to a woman's %VBD take a significant length of time. For this reason, if there is a correlation between these variables, the lack of correlation could have been due to the duration of the study, and there is the potential that if the study were longer, there could be a correlation between the changes with the elasticity values and the %VBD values. Future longitudinal trials, with multiple yearly, follow-ups, could incorporate SWE to quantify breast elasticity as an early measure and then continue with mammography and SWE for the extended length of the trial. A trial of this nature is needed to determine if changes in %VBD correlation with the elasticity but just at a later time point.

The correlation between the change in breast elasticity and change in TFV, from both the HAVAH*T+Ai*™ and the enobosarm and anastrozole study demonstrated consistent results. Within the HAVAH*T+Ai*™ study, the correlation between the change in TFV from baseline to month 12 and change in elasticity at month 1 and 3, both showed a statistically significant, moderate correlation. While the correlation between change in TFV and elasticity at 2 months demonstrated a high correlation, this correlation also reaching statistical significance. The same pattern was observed in the enobosarm and anastrozole study with a moderate correlation between the baseline and 12-month change in TFV and elasticity and a low (r= 0.459) correlation with the change in elasticity at one month, with the r-value threshold for a moderate correlation being 0.500. However, both of these findings did not reach the conventional level of statistical significance.

These results suggest that there is consistency in the two studies showing that breast elasticity decreases with a hormonal intervention that also reduces %VBD and TFV, and that there was a

relationship with the changes between these variables. There was also consistency in the correlation between the changes in TFV and breast elasticity, and these correlations were able to be observed at an early time point within the studies. These findings are favourable for the objective of this research program, as this evidence suggests that breast elasticity responds in a similar manner to the measured mammography variable of TFV, which is a key feature of a biomarker. These breast elasticity responses also occurred within the first few months of using the interventions, which allows the potential of breast elasticity to predict the changes in TFV at an early time point when using a hormonal intervention. The early response is beneficial as clinically, breast elasticity as measured by SWE may be able to determine if a patient is a responder or a non-responder to hormonal interventions which have been prescribed to reduce MBD and aid in the decision making for either the continuation or cessation of treatment. Also, within the realm of research, it could shorten the length of time required for conducting clinical trials, as breast elasticity is more sensitive to demonstrate a response to an intervention and this response occurs within the first few months of treatment.

9.3 The Protocol that Should be Used to Determine Whole Breast Elasticity

From the literature review, it was found that using SWE to measure whole breast elasticity is a relatively new indication, and for this reason there was no consistently used protocol or methodology described in the literature that demonstrated valid and reliable results as the psychometric properties were not recorded. A key objective of this research program was to develop a protocol for this indication to be used in both the clinical and research settings and the development of this protocol was considered to be a priority in this research program. The reason for this priority was that within the studies included in this thesis that used SWE, the methodology needed to produce valid and reliable elastography results. During the initial SWE sessions, there were fluctuating elasticity values within each breast quadrant with the repeated measures; with these fluctuations not appearing to follow any trend or pattern. In addition to this, through experimentation, it was found that the researcher could easily manipulate the data if the Q-Box™ were not placed carefully or consistently on the image. Secondly, there is a need for a protocol that other researchers can use when conducting further SWE studies for whole breast elasticity. For research purposes, and clinical purposes, it is beneficial to use a consistent protocol to produce comparable results; this helps to

contribute to the body of evidence regarding whole breast elasticity and helps monitor a patient's health accurately.

Through this research program, different participant positions and a variety of *post-hoc* imaging analysis techniques were explored. These techniques included placing Q-Box™ with a predefined diameter or tracing a custom Q-Box™ around the full image to create a large ROI. This ROI is the area included in the breast elasticity calculation. To make the decision about which technique should be described as the ideal protocol, several key desirable parameters were considered; these included the ability to generate a valid breast elasticity results, which was an approximation as we did not have a validated reference standard with which to compare the results. However, omitting artefacts and "black holes" may provide more valid results than if these were left within the ROI.

The ideal technique also needed to have a high level of precision, which demonstrated the minimal spread of values of the collected data. Furthermore, another critical parameter that needs to be considered was the clinical utility of the outcome measure, with the technique ideally needing to be conducted in a timely manner and not be significantly labour intensive for the researcher. A further, important additional consideration of the chosen technique should be the comfort levels of the patients; the chosen technique should be relatively comfortable for the patient or participant, as this has the potential to reduce attrition rates as it is already subjectively an uncomfortable experience for some of the participants.

The methodology that was chosen to be described as the standardised technique is described below in Section 9.3.1 to 9.3.4. The justification for why these decisions were made are also presented. This methodology is appropriate for the SuperSonic™ Imagine Aixplorer® SWE ultrasound machine (Figure 9-1) and may differ for other SWE ultrasound devices.

Figure 9-1: SuperSonic™ Imagine Aixplorer® Shear Wave Elastography Ultrasound machine

9.3.1 Patient Position

The advice regarding patient position requires more research to make a definitive statement. From the data analysed in this research program, the suggested position should be the patient lying supine on the examination table with their arms resting relaxed by their side. During this research program, two positions were researched; one with the participants having their arms relaxed by their side and the other position had the participants ipsilateral arm to the breast being examined resting above their head. There was a statistically significant difference of 1.55kPa between the two positions. As previously stated, there is currently no validated gold standard to compare the elasticity values to establish which technique is more accurate. There were two reasons for the decision that arms relaxed was the ideal position of choice for measuring whole breast elasticity. Firstly, the IQR was 3.38 in the arms relaxed group compared to 4.75 in the arm above the head group, showing that the relaxed arms group produced more precise data. The second reason for choosing this position was the subjective interpretation of the body language of the participants, with it appearing that the participants were more comfortable with their arms relaxed by their side.

As suggested, the guidelines for the patient position when using SWE to measure whole breast elasticity requires more research. When using traditional ultrasound, the patient is usually

lying supine with a pillow placed under the shoulder of the breast to be examined, and the patient's arm elevated over their head (Kossoff 2000); this position allows the breast to be distributed over the chest wall. Traditional ultrasound, however, is used to investigate specific regions of the breast, rather than calculate the tissue properties of the whole breast as we have been doing with SWE. In addition to this, breast elasticity can change with gravity-induced deformations that occur with changes in tissue weight distribution (Griesenauer, Weis et al. 2017). So, if using the traditional ultrasound patient position, the rotation of the torso would increase the gravitational forces on the breast and may alter the breast elasticity measurements. The process of elevating the arm may also increase the strain on the breast tissue. This increased strain may contribute to the mean elasticity values being higher in this position, out of the two positions measured, which was seen in the result with this position producing significantly higher elasticity values in the Chapter 8 study.

9.3.2 Transducer Setup and Placement Position

A linear transducer (Figure 9-2) is used, and ample ultrasound gel is placed on the transducer head (Figure 9-3) and also directly on the breast (on each quadrant to be imaged).

Figure 9-2: SuperSonic™ Imagine Aixplorer® SWE linear transducer

Figure 9-3: Ultrasound gel being placed on the linear transducer head

The ample ultrasound gel aids the propagation of the shear waves and reduces the extent of the artefacts and or areas that the shear waves have not propagated (black holes) within the image. In regard to the placement of the transducer head, each breast is divided into four quadrants (Figure 9-4); the lower outer (1, 8), upper outer (2, 7), upper inner (3, 6) and lower inner quadrants (4, 5). The transducer head is placed horizontally in a perpendicular position, 2cm away from the nipple (Figure 9-5).

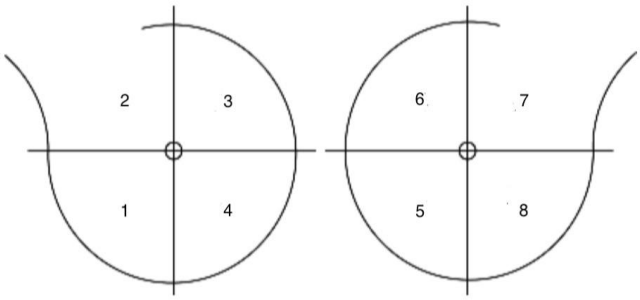

Figure 9-4: Diagram of breast quadrants for shear wave ultrasound images

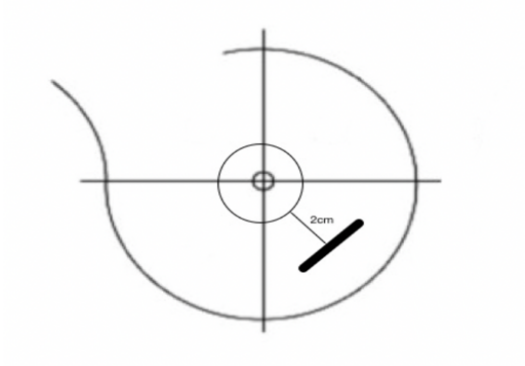

Figure 9-5: Location of the transducer head in relation to the nipple when using SWE

With the ample ultrasound gel, the transducer is held in the position with no pressure applied from the operator for approximately three to five seconds, allowing time for shear waves to propagate through the tissue (this allows for a black hole free image). The image is then frozen and labelled by using a body mark on the image (Figure 9-6).

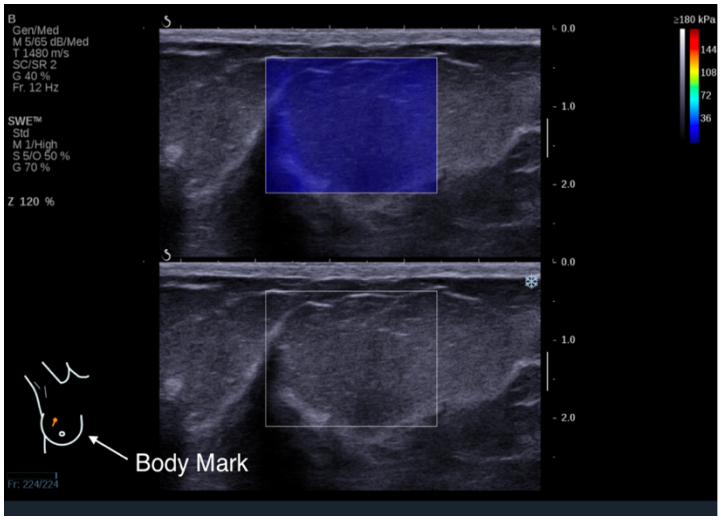

Figure 9-6: SWE output image with body mark indicating what breast quadrant has been imaged

By capturing images of each breast quadrant, it can provide useful information as the elasticity values differ between different quadrants and to establish a thorough calculation of the whole breast elasticity all quadrants need to be included in the analysis. Once the images have been saved, they are analysed *post hoc*.

9.3.3 Post-hoc Analysis of the Shear Wave Elasticity Images

After capturing and saving the SWE images, there needs to be the *post-hoc* analysis to calculate and present the breast elasticity of the ROI. The function on the SuperSonic™

Aixplorer® SWE ultrasound machine that was chosen to do this was the Q-Box™ trace (Figure 9-7).

Figure 9-7: Q-Box™ trace function command button on SuperSonic™ Aixplorer® SWE ultrasound

The Q-Box™ trace feature allows the user to define the ROI by tracing the desired area on the touch screen using the stylus provided with the SWE machine (Figure 9-8). When using the Q-Box™ trace, the aim is to include as much area in the ROI as possible but omit areas that include artefacts or black holes (Figure 9-9).

Figure 9-8: User using the attached stylus tracing around the desired region of interest

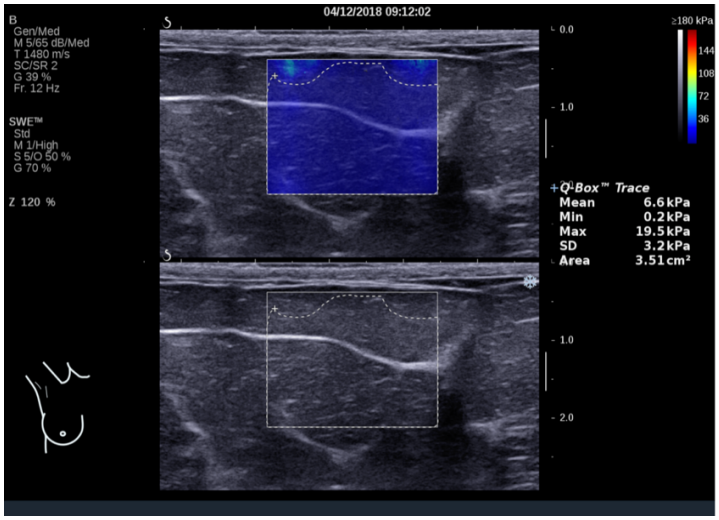

Figure 9-9: Free-Trace Q-Box™ on SWE image to omit ultrasound artefacts

The reasons this method was chosen were due to the analyses in Chapter 6, which include the Q-Box™ trace having the lowest CV, again providing the most precise data which is beneficial for clinical trials to demonstrate a change in the breast elasticity values. This method also took the least amount of time to analyse the images and enter the data into excel due to one dataset per quadrant as opposed to the six datasets that were produced per breast quadrant with some of the other methodologies. Once the data has been entered into the statistical software of choice, the mean, maximum, minimum and SD of the elasticity can be calculated for each quadrant (left and right breast combined), the left or right breast or the average whole breast elasticity, as was done in this research program.

9.4 The Limitations of this Research

As with all research, the studies within this thesis have limitations that need to be considered when interpreting the findings and conclusions.

9.4.1 Pilot Studies and Sample Size

Firstly, both the enobosarm and anastrozole and HAVAH*T+Ai*™ trials were pilot studies, and for this reason, had small sample sizes with no formal power calculations. Therefore, the results produced from the included studies have limited power to detect differences in the results. A pilot study was an appropriate research design for this doctoral research program. The reasoning for this was due to both the included interventions used being novel therapies, and the outcome measure of breast elasticity also being innovative with no published

literature reporting the psychometric properties for its use regarding this indication. Therefore, by conducting a study of this nature, it allowed us to determine if SWE was an appropriate choice of an outcome measure for these studies and we were able to estimate the effect of the enobosarm and anastrozole and HAVAH*T+Ai*™ interventions on breast elasticity. However, as the sample size hasn't been guided by a power calculation, there is the possibility of making inaccurate assumptions or predictions based on these results (Van Teijlingen and Hundley 2001) regarding the validity and the effect of the interventions on breast elasticity. In addition, the future response of breast elasticity in further studies cannot be guaranteed.

The information presented above demonstrates that we need to be cautious about formulating definitive statements regarding the findings of both of these studies. However, both the data from the enobosarm and anastrozole intervention and the HAVAH*T+Ai*™ intervention studies produced statistically significantly higher elasticity values compared to the healthy participant study presented in Chapter 8, and the elasticity decreases and correlations were consistent in both the enobosarm and anastrozole and the HAVAH*T+Ai*™ studies. These results maintain the provide promising data regarding the validity and clinical utility of breast elasticity as a biomarker for MBD.

9.4.2 Uncontrolled Open-Label Study Designs

Both of the intervention studies (enobosarm and anastrozole and HAVAH*T+Ai*™) utilised an uncontrolled, open-label trial design. This open-label trial design specifies that everyone recruited for the study received the active intervention, and this establishes that there was no control group. There are several problems associated with this type of study design. Firstly, as the assessor has not been blinded to the intervention group, the study results can be influenced by observer bias, which is 'any kind of systemic discrepancy from the truth during the process of observing and recording information for a study' (Mahtani, Spencer et al. 2018). Even though the SWE is relatively operator-independent due to the fact no pressure is being placed through the transducer head (unlike in other forms of elastography), this observer bias had the potential to impact the study results. As previously discussed in Chapter 6, the placement of the Q-Box™ can significantly alter the results of the study, so the researcher may have consciously or unconsciously placed the Q-Box™ in a more favourable position to show

better results. We did try and control for this bias by using the Free-Trace Q-Box™, which significantly reduced the opportunities of the researcher to manipulate the date as compared to the other Q-Box™ methodologies. This attempt at controlling for observer bias was unlike other studies which have used SWE for breast tissue analyses, as commonly in these previous studies, the Q-Box™ ratio function was used. The Q-Box™ ratio technique requires the researcher to place two circular Q-Box™ on the image (one Q-Box™ for fibroglandular tissue and one for fatty tissue), which provides the elasticity of the fibroglandular area of the breast. Using the Q-Box™ ratio technique can lead to a substantial level of data manipulation, as only one Q-Box™ is used for the fibroglandular tissue, the researcher can place the Q-Box™ on a region that may provide more favourable results for their hypothesis, whether this be higher or lower elasticity, as the elasticity can fluctuate throughout a single image.

Another limitation of the open-label study design and therefore, the studies included in this research program, is the lack of a control group. By not having a control arm of either the intervention studies, we were unable to compare the breast elasticity changes to a group of participants with similar baseline characteristics, who were not utilising the hormonal interventions used within these trials. The lack of a control group can result in maturation bias affecting the study results, and we are unable to describe the behaviour of the breast elasticity in a population not on the intervention. Therefore, the natural progression of fluctuations or the breast elasticity in the absence of an intervention were unable to be observed and described. We did try to negate for the lack of a control group by incorporating the study from Chapter 8 of this thesis, which used four repeat measure on healthy women to determine the unbiased behaviour of breast elasticity. This study demonstrated no significant differences in the breast elasticity values across the repeat measures or with hormonal fluctuations of the menstrual cycle. However, as the women in the intervention studies were explicitly recruited for having high MBD, and the women in the reliability were recruited from the general local population, there was not a valid comparison group. Any future trials should be designed with a placebo-controlled group, and the assessors should be blinded, which will negate the maturation bias and also reduce the risk of observer bias or any other researcher-based biases that can be introduced.

9.4.3 Inability to Calculate Baseline Correlations Between Breast Elasticity and Per Cent Volumetric Breast Density and Total Fibroglandular Volume

A further limitation of this research program was the inability to conduct a baseline correlation between the whole breast elasticity and %VBD and TFV. As a key hypothesis was that there would be a higher baseline breast elasticity in women with high MBD due to the structure and histology of the tissue. It would have been of value to calculate this correlation to provide fundamental evidence to determine if breast elasticity could be used as a biomarker for MBD. However, during the studies included in this thesis, the only participants that had VolapraDensity™ measurements were the women with high MBD. As we recruited women with no signs of breast disease or pathology for the reliability study in Chapter 8, it was deemed unethical to expose these women to unnecessary radiation which would occur during mammography; therefore, they did not have mammograms and subsequently VolparaDensity™ measurements. For this reason, we were forced to assume that these women had a lower MBD than the women explicitly recruited for their high MBD, due to the reason previously listed in Section 9.2. This allowed us to hypothesise that the baseline elasticity values are higher in women with greater MBD but didn't allow us to make a direct correlation analysis of the spectrum of MBD and breast elasticity.

In future research, it would be beneficial to conduct a cross-sectional study using women from a breast screening program, who have had a mammogram and also then undertake SWE on the breast to measure the elasticity. This would give us a sample of women with varying breast densities to conduct correlation analyses with breast elasticity. If there is a strong correlation between the two variables, this could aid the research into breast elasticity as a biomarker for MBD but could also, when properly validated, be used as a screening tool for women under the age of 40, who are concerned about their breast density. These women are under the age who are eligible for the Australian government-funded breast screening programs; therefore, elasticity may be able to guide the medical practitioner in regard to decision making for whether a woman should be referred for a mammogram.

9.5 Future Research

As previously mentioned, the studies in this thesis are contributing to the early body of evidence for elasticity as a biomarker for interventions to reduce MBD. However, as listed in Section 9.4, there are limitations in the methodologies of the studies within this thesis. There needs to be ongoing research into this area further to validate breast elasticity for use in clinical research. This section will state areas of future research which need to be conducted to further contribute to the literature.

Firstly, normative breast elasticity values measured using SWE need to be accurately measured and validated. This ideally would have a larger cohort, which would be a larger representation of the population, and the averages would be less influenced by individual variations of breast elasticity. In addition, future research needs to measure %VBD, TFV and breast elasticity measurements of all the participants. These measurements, for feasibility reasons during this research program, could not be measured. By doing these additional measures, the researcher will be able to accurately state the normative breast elasticity ranges of individuals with low MBD and high MBD. Furthermore, with all of these breast variables, a linear correlation analysis could be conducted to observe if there is a relationship between breast elasticity and %VBD, and TFV. As mentioned in Section 9.4.3, a potential study design to uncover this data would be a cross-sectional study. The sample for this study could be women recruited from a breast screening program. These women, as a representation of a population of women over 50, would have varying degrees of breast density and TFV, these participants would also undergo SWE to measure their breast elasticity. This mammography data and elasticity data would allow normative values to be calculated, and correlation analyses to be made between the variables. This study could also use a sub-group of women to have their SWE images captured and the corresponding breast elasticity values extracted by two assessors which would allow the inter-rater reliability to be calculated. The inter-rater reliability is a vital psychometric property of an outcome measure for longitudinal studies with repeated measurements and was unable to be calculated within this thesis due to the inability of the researcher to find a second assessor.

Furthermore, as the studies included in this thesis were pilot trials, more robust larger studies and trials need to be conducted. Ideally, this future research should be designed as a double-blind, randomised, placebo-controlled trial. Any future studies of this nature also need to have a sample size calculation done to determine the adequate sample size to allow the findings to be reliable and not due to chance. By running a trial of this nature, the researcher would be able to reduce the risk of bias (for example maturation bias and observation bias) and determine if there was a statistically sound causal relationship between the intervention and the changes in elasticity. In addition, any future works of this nature could have a more regular schedule of assessments, both for SWE and mammography. From designing the research this way, more information regarding what changes are occurring in the breast tissue after the three-month time point and whether mammography variables are also able to detect changes at this early time point could be discovered.

A further plan for supplementary research could be a histological study to determine the structure and histological properties of breast tissue of high and low elasticity values. This research would provide further information into the similarities or differences between the tissue and cellular make up of breasts of high MBD, and breasts of high elasticity and would be incredibly useful to further validate the use of breast elasticity as an outcome measure and a breast cancer screening tool.

9.6 Conclusion

The overall objective of this research program was to determine if breast elasticity, as measured by SWE, could act as a biomarker of the early response of breast tissue to interventions that reduce MBD. Through the clinical trials and subsequent data analysis, this thesis presented early evidence that breast elasticity may prove to be a valid and reliable biomarker to be used in clinical trials. Although the studies were small and of low power, there were significant reductions in the measured breast elasticity, which trended in the same direction of the other MBD variable results, these being a reduction in %VBD and TFV. Also, there is early evidence that baseline elasticity is higher in women with a greater MBD compared to those with a lower MBD. The findings of this research can be the foundation for future research, as we have presented a precise and reliable imaging protocol and evidence that breast elasticity responds to hormonal interventions that have also been shown to

reduce %VBD and TFV. With further research, if proven valid with more extensive clinical trials, breast elasticity could dramatically aid the research into developing novel interventions to reduce MBD. Breast elasticity could also have significant clinical uses to guide health management decisions and provide early feedback into a patient's response to interventions, which could improve health-related outcomes and may improve patient compliance.

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Appendix 1 - Statement of Authorship

Statement of Authorship

Title of Paper	The effect of a subcutaneous combination of Testosterone (T) and Anastrozole (Ai) (HAVAH*T+Ai*™) on VolparaDensity™ automated volumetric Mammographic Breast Density (MBD); an open labelled analysis of clinical practice records.
Publication Status	Unpublished and Unsubmitted work written in manuscript style
Publication Details	No yet published or submitted

Principal Author

Name of Principal Author (Candidate)	Daniella Dougherty		
Contribution to the Paper	Helped manage the data, helped interpret and analyse the data, constructed the manuscript ready for publication		
Overall percentage (%)	40%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Suzanne B Good		
Contribution to the Paper	Constructed database from patient records		
Signature		Date	

Name of Co-Author	Paul Rolan		
Contribution to the Paper	Expert opinion when required for the database contrition and data analysis and interpretation		
Signature		Date	10/06/20

Appendix 1 – Continued

Name of Co-Author	Stephen Birrell		
Contribution to the Paper	Chief medical officer of HAVAH Pty Ltd, provided expert information regarding HAVAH*T+Ai*™ when required. Also, Dr Birrell's patient dataset was used for the analysis.		
Signature		Date	**5/6/20**

Appendix 2 – Visual Analogue Scale for Breast Pain

Patient Name _____
Date _____

BREAST PAIN SCALE

INSTRUCTIONS TO PARTICIPANT

Think about your pain in either breast over the last 4 weeks.

Please mark your answer with an **X** on the horizontal line.

I……I
No Pain **Extreme Pain**

Please note:

a) that the further to the right you place your **X** the **more** pain you are experiencing.
b) that the further to the left you place your **X** the **less** pain you are experiencing.
c) **please do not** place your **X past the end of the line**.

VAS - BREAST PAIN SCALE

Appendix 3 – Menopausal Rating Scale

Patient Name: _____

Date: _____

Menopause Rating Scale (MRS)

Which of the following symptoms apply to you at this time? Please, mark the appropriate box for each symptom. For symptoms that do not apply, please mark 'none'.

	Symptoms:	none	mild	moderate	severe	very severe
	Score =	0	1	2	3	4
1.	Hot flushes, sweating (episodes of sweating)	☐	☐	☐	☐	☐
2.	Heart discomfort (unusual awareness of heart beat, heart skipping, heart racing, tightness)	☐	☐	☐	☐	☐
3.	Sleep problems (difficulty in falling asleep, difficulty in sleeping through, waking up early)	☐	☐	☐	☐	☐
4.	Depressive mood (feeling down, sad, on the verge of tears, lack of drive, mood swings)	☐	☐	☐	☐	☐
5.	Irritability (feeling nervous, inner tension, feeling aggressive)	☐	☐	☐	☐	☐
6.	Anxiety (inner restlessness, feeling panicky)	☐	☐	☐	☐	☐
7.	Physical and mental exhaustion (general decrease in performance, impaired memory, decrease in concentration, forgetfulness)	☐	☐	☐	☐	☐
8.	Sexual problems (change in sexual desire, in sexual activity and satisfaction)	☐	☐	☐	☐	☐
9.	Bladder problems (difficulty in urinating, increased need to urinate, bladder incontinence)	☐	☐	☐	☐	☐
10.	Dryness of vagina (sensation of dryness or burning in the vagina, difficulty with sexual intercourse)	☐	☐	☐	☐	☐
11.	Joint and muscular discomfort (pain in the joints, rheumatoid complaints)	☐	☐	☐	☐	☐

Appendix 4 – Blood Serum Hormone Results

Subject number	Visit	Collection Date	Collection Time	Description	Units	Range Low	Range High	Result	Out of range
001	**Screening**	**3/02/2017**	**0920**	LH	U/L			11.9	
				FSH	U/L			5.1	
				Oestradiol	pmol/L			1006	
				Progesterone	nmol/L			0.3	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<0.6	
				DHEAS	umol/L	1.7	11.7	1.8	
				SHBG	nmol/L	28	150	65	
001	**1 Month**	**07/03/2017**	**0845**	LH	U/L			30.5	
				FSH	U/L			14.2	
				Oestradiol	pmol/L			434	
				Progesterone	nmol/L			1.6	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	4.0	
				DHEAS	umol/L	1.7	11.7	3.4	
				SHBG	nmol/L	28	150	10	*Low
001	**Month 3**	**1/05/2017**	**0850**	LH	U/L			31.1	
				FSH	U/L			12.6	
				Oestradiol	pmol/L			839	

				Progesterone	nmol/L				
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	6.7	
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	9	*LOW
001	**Month 6**	**07/08/17**	**0915**	LH	U/L			1.7	
				FSH	U/L			2.5	
				Oestradiol	pmol/L			118	
				Progesterone	nmol/L			30.0	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<4.0	
				DHEAS	umol/L	1.7	11.7	3.7	
				SHBG	nmol/L	28	150	10	*LOW
001	**Month 9**	**02/11/17**	**0936**	LH	U/L			10.2	
				FSH	U/L			6.3	
				Oestradiol	pmol/L			105	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<4.4	
				DHEAS	umol/L	1.7	11.7	3.6	
				SHBG	nmol/L	28	150	9	*LOW
001	**Month 12**	**05/02/18**	**1220**	LH	U/L			1.5	
				FSH	U/L			2.8	

				Oestradiol	pmol/L			132	
				Progesterone	nmol/L			19.2	
				Testosterone	nmol/L	0	1.8	<0.1	
				FAI	%	0.4	6.0	<0.8	
				DHEAS	umol/L	1.7	11.7	2.9	
				SHBG	nmol/L	28	150	12	*LOW
002	**Screening**	**10/02/2017**	**0815**	LH	U/L			4.1	
				FSH	U/L			2.4	
				Oestradiol	pmol/L			1530	
				Progesterone	nmol/L			1.0	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	0.3	*
				DHEAS	umol/L	1.7	11.7	3.5	
				SHBG	nmol/L	28	150	149	
002	**Month 1**	**15/03/2017**	**Unknown**	LH	U/L			3.4	
				FSH	U/L			6.3	
				Oestradiol	pmol/L			65	
				Progesterone	nmol/L			0.8	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<3.1	
				DHEAS	umol/L	1.7	11.7	4.4	
				SHBG	nmol/L	28	150	13	*LOW
002	**Month 3**	**08/05/2017**	**0920**	LH	U/L			2.7	

				FSH	U/L			7.8	
				Oestradiol	pmol/L			100	
				Progesterone	nmol/L			0.5	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<4.4	
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	9	*LOW
002	**Month 6**	**16/08/2017**	**0800**	LH	U/L			4.2	
				FSH	U/L			2.9	
				Oestradiol	pmol/L			198	
				Progesterone	nmol/L			0.4	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<4.4	
				DHEAS	umol/L	1.7	11.7	3.9	
				SHBG	nmol/L	28	150	9	*LOW
002	**Month 9**	**15/11/17**	**1458**	LH	U/L			46.2	
				FSH	U/L			22.4	
				Oestradiol	pmol/L			526	
				Progesterone	nmol/L			0.6	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<3.3	
				DHEAS	umol/L	1.7	11.7	3.7	
				SHBG	nmol/L	28	150	12	*LOW

002	**Month 12**	**14/02/18**	**1030**	LH	U/L			8.2	
				FSH	U/L			9.6	
				Oestradiol	pmol/L			351	
				Progesterone	nmol/L			0.4	
				Testosterone	nmol/L	0	1.8	<0.1	
				FAI	%	0.4	6.0	<1.0	
				DHEAS	umol/L	1.7	11.7	3.7	
				SHBG	nmol/L	28	150	10	*LOW
003	**Screening**	**07/02/2017**	**0935**	LH	U/L			4.7	
				FSH	U/L			5.0	
				Oestradiol	pmol/L			269	
				Progesterone	nmol/L			14.0	
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	0.8	
				DHEAS	umol/L	1.7	11.7	4.3	
				SHBG	nmol/L	28	150	71	
003	**Month 1**	**07/03/2017**	**0915**	LH	U/L			2.0	
				FSH	U/L			2.7	
				Oestradiol	pmol/L			109	
				Progesterone	nmol/L			10.1	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	4.0	
				DHEAS	umol/L	1.7	11.7	5.4	

				SHBG	nmol/L	28	150	10	*LOW
003	**Month 3**	**03/05/2017**	**0910**	LH	U/L			0.5	
				FSH	U/L			2.1	
				Oestradiol	pmol/L			117	
				Progesterone	nmol/L			11.1	
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	6.7	*HIGH
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	9	*LOW
003	**Month 6**	**10/08/2017**	**0920**	LH	U/L			4.7	
				FSH	U/L			6.8	
				Oestradiol	pmol/L			<50	
				Progesterone	nmol/L			1.4	
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	6.7	*HIGH
				DHEAS	umol/L	1.7	11.7	5.9	
				SHBG	nmol/L	28	150	9	*LOW
003	**Month 9**	**6/11/2017**	**0939**	LH	U/L			8.0	
				FSH	U/L			5.8	
				Oestradiol	pmol/L			89	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	6.7	

				DHEAS	umol/L	1.7	11.7	5.6	
				SHBG	nmol/L	28	150	9	
003	**Month 12**	**09/02/2018**	**0925**	LH	U/L			4.7	
				FSH	U/L			8.9	
				Oestradiol	pmol/L			65	
				Progesterone	nmol/L			0.5	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<4.0	
				DHEAS	umol/L	1.7	11.7	5.8	
				SHBG	nmol/L	28	150	10	CS * LOW
005	**Screening**	**04/02/2017**	**0910**	LH	U/L			5.7	
				FSH	U/L			2.4	
				Oestradiol	pmol/L			331	
				Progesterone	nmol/L			2.6	
				Testosterone	nmol/L	0	1.8	0.7	
				FAI	%	0.4	6.0	0.7	
				DHEAS	umol/L	1.7	11.7	2.1	
				SHBG	nmol/L	28	150	100	
005	**Month 1**	**08/03/2017**	**1050**	LH	U/L			6.6	
				FSH	U/L			7.8	
				Oestradiol	pmol/L			94	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	0.6	

				FAI	%	0.4	6.0	3.5	
				DHEAS	umol/L	1.7	11.7	3.0	
				SHBG	nmol/L	28	150	17	*LOW
005	**Month 3**	**27/04/2017**	**1035**	LH	U/L			3.9	
				FSH	U/L			5.6	
				Oestradiol	pmol/L			136	
				Progesterone	nmol/L				
				Testosterone	nmol/L	0	1.8	0.7	
				FAI	%	0.4	6.0	4.7	
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	15	*LOW
005	**Month 6**	**17/07/2017**	**1425**	LH	U/L			3.8	
				FSH	U/L			3.1	
				Oestradiol	pmol/L			773	
				Progesterone	nmol/L			0.5	
				Testosterone	nmol/L	0	1.8	0.9	
				FAI	%	0.4	6.0	6.0	
				DHEAS	umol/L	1.7	11.7	3.4	
				SHBG	nmol/L	28	150	15	*LOW
005	**Month 9**	**06/11/2017**	**0926**	LH	U/L			6.0	
				FSH	U/L			5.6	
				Oestradiol	pmol/L			479	
				Progesterone	nmol/L			<0.5	

				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<3.1	
				DHEAS	umol/L	1.7	11.7	3.1	
				SHBG	nmol/L	28	150	13	*LOW
005	**Month 12**	**30/01/2018**	**0918**	LH	U/L			7.3	
				FSH	U/L			3.9	
				Oestradiol	pmol/L			506	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	0.5	
				FAI	%	0.4	6.0	3.3	
				DHEAS	umol/L	1.7	11.7	2.7	
				SHBG	nmol/L	28	150	15	*LOW
006	**Screening**	**04/02/2017**	**0000**	LH	U/L			6.8	
				FSH	U/L			6.9	
				Oestradiol	pmol/L			170	
				Progesterone	nmol/L			0.7	
				Testosterone	nmol/L	0	1.8	1.1	
				FAI	%	0.4	6.0	1.5	
				DHEAS	umol/L	1.7	11.7	6.6	
				SHBG	nmol/L	28	150	72	
006	**Month 1**	**03/03/2017**	**0840**	LH	U/L			4.4	
				FSH	U/L			9.0	
				Oestradiol	pmol/L			81	

				Progesterone	nmol/L			0.6	
				Testosterone	nmol/L	0	1.8	1.2	
				FAI	%	0.4	6.0	13.3*	HIGH
				DHEAS	umol/L	1.7	11.7	9.8	
				SHBG	nmol/L	28	150	9	*LOW
006	**Month 3**	**28/04/2017**	**0800**	LH	U/L			3.7	
				FSH	U/L			6.7	
				Oestradiol	pmol/L			220	
				Progesterone	nmol/L			0.7	
				Testosterone	nmol/L	0	1.8	1.3	
				FAI	%	0.4	6.0		
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	6	*LOW
006	**Month 6**	**02/08/2017**	**0910**	LH	U/L			3.5	
				FSH	U/L			6.0	
				Oestradiol	pmol/L			78	
				Progesterone	nmol/L			6.0	
				Testosterone	nmol/L	0	1.8	0.9	
				FAI	%	0.4	6.0	18.0	
				DHEAS	umol/L	1.7	11.7	9.8	
				SHBG	nmol/L	28	150	5	*LOW
006	**Month 9**	**06/11/2017**	**1020**	LH	U/L			2.9	
				FSH	U/L			4.4	

				Oestradiol	pmol/L			287	
				Progesterone	nmol/L			0.2	
				Testosterone	nmol/L	0	1.8	0.8	
				FAI	%	0.4	6.0	13.3*	High
				DHEAS	umol/L	1.7	11.7	8.3	
				SHBG	nmol/L	28	150	6*	LOW
006	**Month 10**	**29/12/2017**	**1130**	LH	U/L			3.9	
				FSH	U/L			6.5	
				Oestradiol	pmol/L			135	
				Progesterone	nmol/L			3.9	
				Testosterone	nmol/L	0	1.8	0.9	
				FAI	%	0.4	6.0	15.0	*HIGH
				DHEAS	umol/L	1.7	11.7	10	
				SHBG	nmol/L	28	150	6	*LOW
008	**Screening**	**06/02/2017**	**0915**	LH	U/L			3.3	
				FSH	U/L			6.4	
				Oestradiol	pmol/L			187	
				Progesterone	nmol/L			54.8	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	0.5	
				DHEAS	umol/L	1.7	11.7	3.4	
				SHBG	nmol/L	28	150	78	
008	**Month 1**	**07/03/2017**	**1240**	LH	U/L			3.2	

				FSH	U/L			4.9	
				Oestradiol	pmol/L			119	
				Progesterone	nmol/L			22.6	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<3.1	
				DHEAS	umol/L	1.7	11.7	5.2	
				SHBG	nmol/L	28	150	13	*LOW
008	**Month 3**	**04/05/2017**	**0000**	LH	U/L			5.4	
				FSH	U/L			7.6	
				Oestradiol	pmol/L			66	
				Progesterone	nmol/L			3.8	
				Testosterone	nmol/L	0	1.8	0.6	
				FAI	%	0.4	6.0	6.7	*HIGH
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	9	*LOW
008	**Month 6**	**04/08/2017**	**1037**	LH	U/L			7.2	
				FSH	U/L			14.6	
				Oestradiol	pmol/L			<44	
				Progesterone	nmol/L				
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	5.0	
				DHEAS	umol/L	1.7	11.7	5.3	
				SHBG	nmol/L	28	150	8	*LOW

008	**Month 9**	**14/11/2017**	**0000**	LH	U/L			4.0	
				FSH	U/L			5.0	
				Oestradiol	pmol/L			94	
				Progesterone	nmol/L			6.2	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<3.3	
				DHEAS	umol/L	1.7	11.7	4.9	
				SHBG	nmol/L	28	150	12	*LOW
008	**Month 12**	**06/02/2018**	**1113**	LH	U/L			6.1	
				FSH	U/L			13.5	
				Oestradiol	pmol/L			61	
				Progesterone	nmol/L			0.3	
				Testosterone	nmol/L	0	1.8	0.5	
				FAI	%	0.4	6.0	2.8	
				DHEAS	umol/L	1.7	11.7	6.3	
				SHBG	nmol/L	28	150	18	*LOW
010	**Screening**	**06/03/2017**	**0855**	LH	U/L			1.1	
				FSH	U/L			1.0	
				Oestradiol	pmol/L			<44	
				Progesterone	nmol/L			0.3	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	0.1	
				DHEAS	umol/L	1.7	11.7	1.9	

				SHBG	nmol/L	28	150	281	*HIGH
010	**Month 1**	**07/04/2017**	**1040**	LH	U/L			2.5	
				FSH	U/L			2.4	
				Oestradiol	pmol/L			182	
				Progesterone	nmol/L			36.8	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	1.7	
				DHEAS	umol/L	1.7	11.7	3.9	
				SHBG	nmol/L	28	150	24	*LOW
010	**Month 3**	**30/05/2017**	**0950**	LH	U/L			8.0	
				FSH	U/L			3.9	
				Oestradiol	pmol/L			254	
				Progesterone	nmol/L			0.4	
				Testosterone	nmol/L	0	1.8	0.5	
				FAI	%	0.4	6.0	3.6	
				DHEAS	umol/L	1.7	11.7	4.3	
				SHBG	nmol/L	28	150	14	*LOW
010	**Month 6**	**13/09/2017**	**1030**	LH	U/L			4.4	
				FSH	U/L			9	
				Oestradiol	pmol/L			127	
				Progesterone	nmol/L			2	
				Testosterone	nmol/L	0	1.8	0.26	
				FAI	%	0.4	6.0	1.5	

				DHEAS	umol/L	1.7	11.7	4.2	
				SHBG	nmol/L	28	150	17	
010	**Month 9**	**04/12/2017**	**0930**	LH	U/L			7.1	
				FSH	U/L			5.9	
				Oestradiol	pmol/L			69	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	<0.1	
				FAI	%	0.4	6.0	<0.7	
				DHEAS	umol/L	1.7	11.7	4.3	
				SHBG	nmol/L	28	150	15	*LOW
010	**Month 12**	**13/03/2018**	**1400**	LH	U/L			4.0	
				FSH	U/L			4.1	
				Oestradiol	pmol/L			275	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<2.9	
				DHEAS	umol/L	1.7	11.7	5.5	
				SHBG	nmol/L	28	150	14	*LOW CS
011	**Screening**	**11/03/2017**	**0933**	LH	U/L			3.1	
				FSH	U/L			6.8	
				Oestradiol	pmol/L			128	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	0.2	

				FAI	%	0.4	6.0	0.5	
				DHEAS	umol/L	1.7	11.7	3.9	
				SHBG	nmol/L	28	150	40	
011	**Month 1**	**06/04/2017**	**0945**	LH	U/L			3.1	
				FSH	U/L			9	
				Oestradiol	pmol/L			pending	
				Progesterone	nmol/L			pending	
				Testosterone	nmol/L	0	1.8	0.54	
				FAI	%	0.4	6.0	7.7	
				DHEAS	umol/L	1.7	11.7		
				SHBG	nmol/L	28	150	7	
011	**Month 3**	**19/06/2017**	**0920**	LH	U/L			4.0	
				FSH	U/L			8.5	
				Oestradiol	pmol/L			83	
				Progesterone	nmol/L			0.7	
				Testosterone	nmol/L	0	1.8	<0.4	
				FAI	%	0.4	6.0	<1.8	
				DHEAS	umol/L	1.7	11.7	4.6	
				SHBG	nmol/L	28	150	22	*LOW
011	**Month 6**	**12/09/2017**	**1130**	LH	U/L			5.5	
				FSH	U/L			3.7	
				Oestradiol	pmol/L			141	
				Progesterone	nmol/L			19.0	

				Testosterone	nmol/L	0	1.8	0.5	
				FAI	%	0.4	6.0	10.0	
				DHEAS	umol/L	1.7	11.7	6.6	
				SHBG	nmol/L	28	150	5	*LOW
011	**Month 9**	**07/12/2017**	**1032**	LH	U/L			4.5	
				FSH	U/L			6.8	
				Oestradiol	pmol/L			120	
				Progesterone	nmol/L			<0.5	
				Testosterone	nmol/L	0	1.8	<0.1	
				FAI	%	0.4	6.0	<1.4	
				DHEAS	umol/L	1.7	11.7	5.3	
				SHBG	nmol/L	28	150	7	*LOW
011	**Month 12**	**13/03/18**	**1420**	LH	U/L			1.6	
				FSH	U/L			1.8	
				Oestradiol	pmol/L			124	
				Progesterone	nmol/L			12.0	
				Testosterone	nmol/L	0	1.8	0.4	
				FAI	%	0.4	6.0	5.0	
				DHEAS	umol/L	1.7	11.7	6.4	
				SHBG	nmol/L	28	150	8	*LOW

Appendix 5 – Statement of Authorship

Statement of Authorship

Title of Paper	The behaviour of breast elasticity as measured by Shear Wave Elastography in healthy women in regard to menstrual cycle changes, repeatability and intra-rater reliability.
Publication Status	**Unpublished and Unsubmitted work written in a manuscript style**
Publication Details	**Not yet published or submitted**

Principal Author

Name of Principal Author (Candidate)	Daniella Dougherty		
Contribution to the Paper	Created the research protocol, applied for ethics, recruited all of the participants, conducted all the data collection, entry and analysis and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	**31/5/2020**

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Stephen Birrell		
Contribution to the Paper	Provided guidance for the analysis of the menstrual cycle hormones and expert guidance for the analysis of the shear wave elasticity data		
Signature		Date	**5/6/20**

Name of Co-Author	Professor Paul Rolan		
Contribution to the Paper	Provided guidance for the ethics application and recruitment of the participants. Provided guidance for the statistical analysis of the data. Provided guidance with the writing of the manuscript.		
Signature		Date	10/06/20