Imaging of tumour microvasculature using high-resolution contrast-enhanced ultrasound together with markers of proliferation, angiogenesis and vascular mimicry to characterise the response to neoadjuvant chemotherapy in triple-negative breast cancer.

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Declaration:

No part of this thesis has been submitted to support an application for any degree or qualification of the University of Kent or any other University or institute of learning.

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Finally, thank you to Baba Ji. So satgur pyara mere naal hai, all I have achieved is down to your blessings.

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Abbreviations

B-mode	Brightness mode
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CC	Craniocaudal
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
CEUS	Contrast-enhanced ultrasound
CISR	Cisplatin-resistant cell line
CK14	Cytokeratin 14
CK5	Cytokeratin 5
CK6	Cytokeratin 6
DAB	3,3' - Diaminobenzidine
DPX	Dibutylphthalate polystyrene xylene
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
FBS	Fetal bovine serum
H&E	Haematoxylin and eosin
HER2	Human epidermal growth factor 2
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IMDM	Iscove's modified dulbecco's medium
Ki67	Antigen Ki-67
MAbs	Monoclonal antibodies
MDT	Multi-disciplinary team meeting
MLO	Medial lateral oblique
MMG	Mammogram
MTW	Maidstone & Tunbridge Wells

NACT	Neoadjuvant chemotherapy
PAS	Periodic acid-Schiff
PBS	phosphate-buffered saline
pCR	Pathological complete response
PD-L1	Programmed death-ligand 1
PET FDG	Positron emission tomography fluorodeoxyglucose
PR	Progesterone receptor
ROI	Region of interest
SF_6	Sulphur hexafluoride
TNBC	Triple-negative breast cancer
USS	Ultrasound
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VM	Vascular mimicry

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Abstract

Triple-negative breast cancer (TNBC) represents 10-15% of all breast cancer cases diagnosed annually in the UK. Treatment frequently involves neoadjuvant chemotherapy, immunotherapy and surgical excision. Current imaging techniques to monitor and predict disease response have limited accuracy. New high-resolution contrast-enhanced ultrasound (CEUS) techniques have the ability to image gross tumour morphology and its microvasculature to visualise angiogenesis.

This research aimed to characterise microvessel density (a surrogate marker for angiogenic activity) as quantified by imaging features using high-resolution CEUS and biomarkers of proliferation, angiogenesis and vasculogenic mimicry during neoadjuvant chemotherapy (NACT). The study also investigated whether germline mutations, as well as the basal type phenotype, correlated with microvessel density and tumour response.

Two patients with triple-negative breast cancer and planned neoadjuvant treatment were recruited and, at specific points of NACT, underwent high-resolution CEUS and immunohistochemistry of tissue samples to assess their response to NACT. Both patients (TNBC001 and TNBC002) demonstrated an encouraging response during neoadjuvant treatment radiologically and histologically. TNBC001 has been found to have complete pathological response, and TNBC002 is awaiting completion of the research study.

This preliminary study indicates that high-resolution CEUS is a potentially valuable tool to monitor treatment response during NACT in patients with TNBC with a demonstrable reduction in microvessel density. The results presented here provide the evidence required to initiate a larger-scale study, which will be needed, to change clinical practice.

Chapter 1: Introduction

1.1 Cancer

Cancer is a broad term used to define a disease process in which there is uncontrolled growth of cells, often with the invasion of healthy tissues locally or throughout the body (1). In normal physiology, cell division is under strict control, where old or damaged cells undergo programmed cell death (apoptosis), and new cells are formed. This orderly programme breaks down in the cancerous setting, and cells can multiply without regulation. These unregulated cells may spread and invade nearby or distant structures, which can affect the function of the invaded structures; as these cancer cells proliferate, they become increasingly abnormal and require more of the body's metabolic output for their growth and development—damage caused by their invasion results in organ malfunction, pain and often death.

Cancer is the second leading cause of death globally (2), accounting for 9.6 million deaths worldwide. Cancer represents a significant public health risk. Thus, intense research effort is still needed to mitigate its effect.

1.1.1 Breast cancer

Breast cancer is the most common cause of cancer in women in the UK. One in seven women in the UK will develop breast cancer in their lifetime (3). In the UK, a third of breast cancer occurs in females over the age of 70, with less than 1 in 5 occurring in those under 50 (4). Breast cancer is a diverse group of diseases and may reflect invasive or non-invasive (in-situ cancer). Invasive breast cancers may also be further subdivided via the expression of specific proteins, these include the oestrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor 2

receptor (HER2). Breast cancers positive for the expression of these proteins are treated as hormone-sensitive whilst those negative for the expression are labelled as triple-negative breast cancer (TNBC)(5). TNBC comprises 10-15% of all new breast cancer diagnoses annually in the UK (6). This disease represents a clinical challenge as whilst it represents a small proportion of overall new breast cancer diagnoses, it has the worst prognosis of all breast cancer types secondary to its lack of expression of these three treatable drug targets. At diagnosis, TNBC tumours are often larger, less differentiated, have an increased likelihood to metastasise, have an increased potential for recurrence within five years (7) and generally affect women under 50 years old, many of whom are in their reproductive years or have young families. In addition, TNBC tumours demonstrate significant chromosomal instability attributed to a defect in DNA repair pathways, specifically homologous recombination (8), germline mutations in breast cancer gene 1 *(BRCA1*) and breast cancer gene 2 (*BRCA2*) in 10-20% of TNBC tumours, with somatic mutations occurring in 3-5% of cases (9).

1.1.2 TNBC treatment

In the UK, the mainstay of treatment involves neoadjuvant chemotherapy (NACT) followed by surgical excision. Histological evidence of final surgical specimens has demonstrated that up to 50% of patients with TNBC tumours achieve pathological complete response (pCR) at the time of surgery (10). Oncologists frequently adopt an intense chemotherapeutic approach with sequential anthracycline and taxane regimens, and the use of platinum agents is associated with an increase in pCR (11,12).

More recently, the National Institute of Clinical Excellence (NICE) has recommended the use of the immunotherapy drug (pembrolizumab) as an option for some patients with TNBC. Those patients with TNBC who have been deemed suitable are those with

early TNBC at high risk of recurrence or locally advanced TNBC that has not spread elsewhere (13). This has been shown to increase the pCR to around 60% from baseline (14).

Conventional imaging measures such as B-mode ultrasound, mammography and magnetic resonance imaging (MRI) are commonly used to monitor disease response to treatment in the breast. Together they have a high sensitivity to detect residual disease (approximately 96%) but a low specificity (approximately 26.7%). (10) Consequently, the Association of Breast Surgery (ABS) have recently established research priorities; one of which is to establish the optimal modality for breast cancer patients undergoing NACT and assess for a response without surgical intervention (5).

1.1.3 Existing knowledge

TNBC shows significant heterogeneity (15), and various groups have attempted to classify them based on gene expression profiles, chemotherapy response, and DNA or RNA profiling. An additional classification system is based on the presence of one or more of the basal cytokeratins (CK5/6 and CK14) to classify them as basal-like (16,17).

Ki67 is a nuclear antigen that can be used as a surrogate marker of cell proliferation in both the normal and cancerous cell (18). In breast cancer in particular it has been shown that it is present in very low levels and exclusively in ER-negative cells therefore it can be used as an ideal marker in TNBC as a sign of proliferation. Previous studies have acknowledged that Ki67 alone has limited clinical validity at present as it is not used routinely in the clinical setting (18)

High expression of EGFR in breast cancer is associated with a high proliferation rate and EGFR expression is more common in younger women and associated with lower

hormone receptor (ER/PR) levels and genomic instability(19). Aberrant EGFR signalling promotes tumour cell migration and survival (20). The presence of lymphatics and blood vessels within and around malignant tumours plays a role in cancer progression, as does the formation of new blood vessels (angiogenesis). Microvessel density is a measure of angiogenesis and is commonly quantified by counting blood vessels in tumour sections stained with vascular markers such as CD34/ CD31 (21). The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are also frequently overexpressed in TNBC and promote changes in vascular endothelial cells, the basement membrane and the surrounding extracellular matrix. Co-expression of EGFR and VEGFR enhances tumour growth and angiogenesis in an autocrine and paracrine manner respectively (22).

Basal like breast cancer and TNBC demonstrate higher microvessel density versus other breast cancer types but microvessel density is not associated with overall survival (21). Several monoclonal antibodies (mAbs) targeting the VEGF/VEGFR signalling pathways can be used to treat a variety of cancers but ramucirumab (which represents one such mAb) targets VEGFR2 did not show a statistically significant improvement in outcome in locally recurrent or metastatic breast cancer (23).

An alternative tumour microcirculation method is vascular mimicry (VM). This is distinct from classical angiogenesis in that it does not depend on endothelial cells and is characterised by the absence of vascular endothelial cells on the inner wall of the blood vessel, has vascular-like channels which are lined by tumour cells and are positive for periodic acid-Schiff (PAS) staining but negative for CD31 staining (24). VM is associated with poor overall survival in breast cancer patients, and anti-angiogenic treatment of TNBC may even facilitate this pathway (25).

1.1.4 Breast Imaging

The main imaging modalities used for the early detection of breast cancer in the UK include mammography (MMG) and ultrasonography (USS). MMG involves using X-rays to look at breast cancers that are sometimes too small to be seen or felt; as such, it has an essential role in early breast cancer detection and is utilised in many countries worldwide as a screening tool. MMG is not always feasible/ appropriate, especially in patients with dense breast tissue and younger patients with an overall reduced risk of breast cancer (26). USS is often the first line imaging modality in younger patients and those with dense breast tissue (27). It can provide information regarding the morphology, orientation, structure and borders of the breast cancer (28).

Standard USS in the UK consists of the use of brightness mode ultrasound . This displays a cross-sectional image that is constructed from echoes that are generated by the reflection of ultrasound waves at tissue boundaries and scattering from small irregularities within tissues. The image's brightness is related to the strength or amplitude of the echo; therefore it is known as brightness mode or B-mode USS (29). To form the image, a source of ultrasound (the transducer) is placed in contact with the skin, and short bursts/ pulses are sent into the patient. These are directed along narrow beam-shaped paths, and as they travel, they are reflected and scattered and generate echoes, some of which travel back to the transducer, where they are detected and used to form the image (**Figure 1**). This form of USS can give valuable information for early breast cancer diagnosis and this has a relatively high sensitivity of 76% and specificity of 84% (30). This form of USS cannot give information regarding the tumour vessel density, which can be proportional to the tumour size and pathological severity (31).

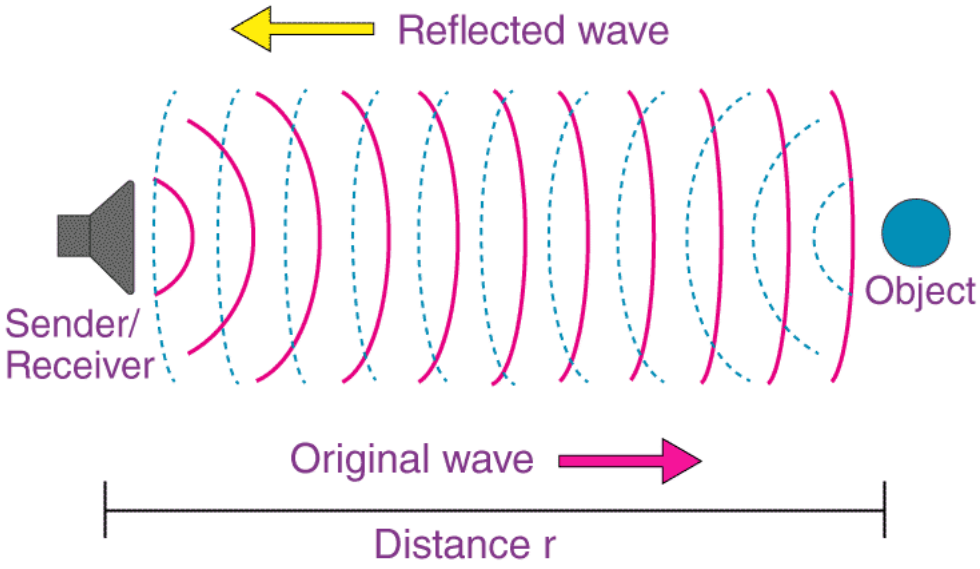


***Figure 1 Ultrasound function**. Ultrasonic waves produced by a transducer and progress until encountering an object of interest and are reflected back to a transducer to create an electrical signal to create a two-dimensional image of tissue and organs (32)*

Contrast-enhanced ultrasound (CEUS) can be used to give such vessel density information. In this modality intravenously injected gas microbubbles provide information regarding the vasculature surrounding the breast cancer. Microbubbles are gas-encapsulated shells with diameters ranging from 1 µm – 7 µm and are more echogenic than red blood cells and are confined to the intravascular spaces. A commonly used contrast agent is sulphur hexafluoride (SF_6) which is stabilised in a phospholipid shell. The difference in acoustic impedance and compressibility between the microbubbles and surrounding media results in non-linear scatters and when combined with nonlinear imaging techniques demonstrates information regarding the vascular morphology (28).

In animal models, the combination of high-resolution USS and CEUS has allowed for detailed imaging of the microvasculature. High-resolution USS offers up to tens of

thousands of imaging frames per second, significantly improving CEUS and reducing artefacts (33) (**Figure 2**).

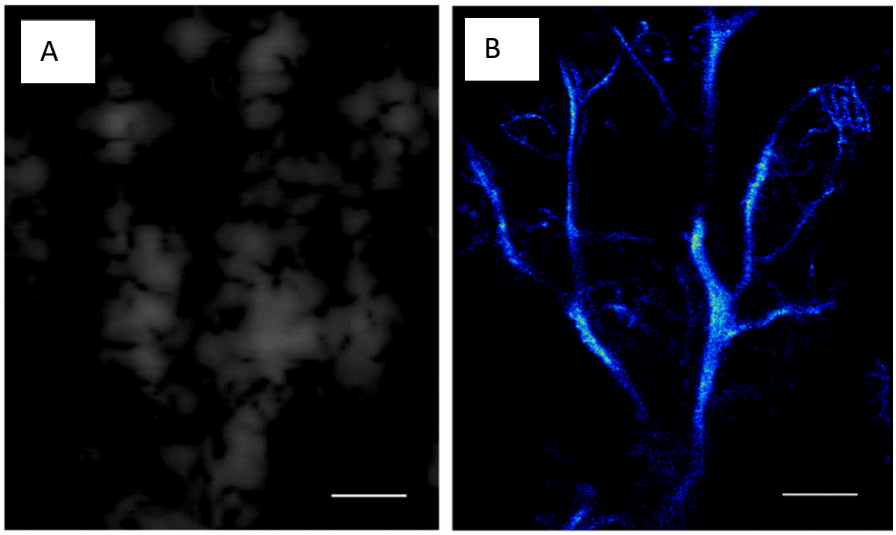


Figure 2 Comparison of CEUS and High-resolution CEUS. *Demonstrating improved imaging potential through increased frames per second and a reduction in artefacts. (A) Standard CEUS – utilising microbubbles as contrast material to visualise vasculature with standard ultrasonography ; (B) High-resolution CEUS – utilising microbubbles as contrast material with high-resolution ultrasonography (33)*

1.1.5 Immunohistochemistry

Immunohistochemistry (IHC) has been utilised in surgical pathology for nearly half a century (34) and in particular in breast pathology, it has become essential for providing prognostic and predictive information. In its most basic form, IHC uses antibodies to detect the location of proteins and other antigens in tissue sections. The antibody-antigen interaction can then be visualised through various methods. Therefore, a robust, optimised, reproducible staining regimen is required.

The basic steps in IHC involve: (**Figure 3**)

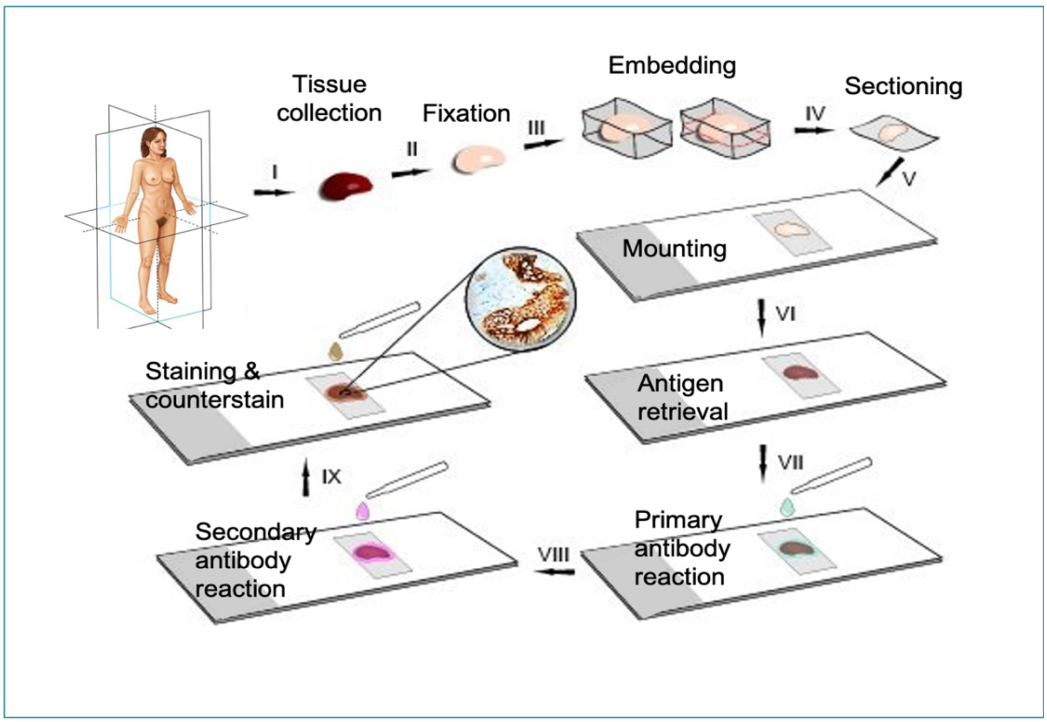


***Figure 3 IHC principles**. 9 steps involved in most IHC performed (34). Comprising tissue collection, fixation, embedding, sectioning, mounting, antigen retrieval, primary antibody reaction, secondary antibody reaction, staining and counterstaining. This culminates in visualisation and assessment.*

1) Tissue collection – This can be from core biopsies or final surgical specimens.
2) Fixation – This involves placing the sample in formalin which fixes the tissue and forms cross-linkages between reactive amino groups
3) Embedding – This involves placing the sample in wax where the fixation products are replaced by this wax through a series of dehydration steps
4) Sectioning – This used a manual/ automatic microtome to slice the sections of the block into an appropriate thickness
5) Mounting – The sliced sections are then placed onto a charged glass slide
6) Antigen retrieval and blocking – This involves blocking endogenous enzymes and antibodies and minimises background staining to reduce false positives
7) Primary antibody reaction – involves exposing the sections to the antibody of interest for a set length of time

8) Secondary antibody reaction – involves exposing the section to a secondary antibody raised against the primary antibody of interest
9) Staining and counterstaining – involves applying an appropriate substrate that may promote a coloured reaction product that can be visualised under a microscope.

Breast tissue is composed of a luminal epithelial cell layer, which is enveloped by myoepithelial cells and encompassed by a basement membrane (**Figure 4)** (35). The recognition of the loss of myoepithelial boundary is sufficient to demonstrate the presence of invasive carcinoma. This can sometimes prove difficult when using haematoxylin and eosin (H&E) stains only and therefore, the use of specific antibody markers can be invaluable when interpreted together. In the case of TNBC as aforementioned, they do not display the ER, PR or HER2 antigens.

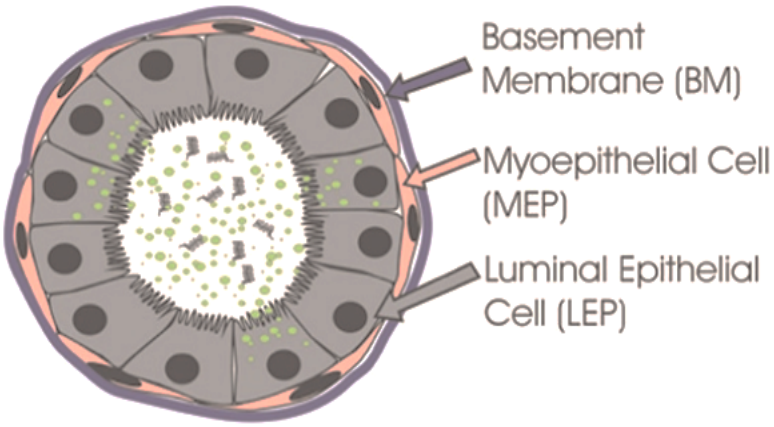


Figure 4 Cross section of a bilayered breast duct. *Loss of myoepithelial boundary denotes the presence of invasive breast cancer as this demonstrates a breach of basement membrane and infiltration of surrounding stroma (35)*

1.2 Rationale for study

Hypothesis:

The combined use of high-resolution CEUS and immunohistochemical markers of angiogenesis (CD34, CD31, VEGF, VEGF-R), proliferation (EGFR, Ki67) and vasculogenic mimicry (VM) (H&E, PAS staining, absence of CD31) will provide a more accurate way to monitor TNBC tumours versus standard imaging in patients undergoing neoadjuvant chemotherapy (NACT) and will provide improved specificity for pCR.

This is a clinical feasibility study with the aim of progressing to larger trials to determine whether this approach will identify patients who may avoid surgical intervention altogether versus those with early progression and require NACT regimen changes or expedited surgical intervention.

Aim:

This clinical study in human subjects aims to utilise high-resolution CEUS as a tool to image the microvasculature of triple-negative breast cancer (TNBC) tumours in patients undergoing neoadjuvant chemotherapy (NACT) and correlate the imaging with established markers of angiogenesis, proliferation and vascular mimicry (VM).

1.2.1 Study objectives

- To investigate whether imaging changes in the tumour microvasculature are an indication of a positive response to NACT and are reflected in the decreased expression of proliferative markers, markers of angiogenesis and vascular mimicry (VM).

- To investigate whether overall disease response and changes in the microvasculature are influenced by the basal type phenotype or mutations in *BRCA 1/2*.

1.2.2 Participant entry

Inclusion criteria

1. Provision of written informed consent (**Appendix 6.1**)
2. Histologically confirmed primary TNBC larger than 20mm with planned NACT.
3. Biologically Female aged 18 to 60 years.
4. In the Investigator's opinion, adhering to the trial recommendations and governance.

Exclusion criteria:

1. Previous ipsilateral breast cancer treated with radiotherapy or chemotherapy.
2. Participant who is pregnant, lactating or planning pregnancy during the course of the study.
3. Allergy to ultrasound contrast.
4. Cannot provide consent.
5. Inflammatory or locally advanced breast cancer.
6. Metastatic breast cancer.
7. TNBC subtype associated with good prognosis (adenoid cystic carcinoma, secretory carcinoma, acinic cell carcinoma, carcinoma with apocrine

differentiation, low-grade metaplastic carcinoma and carcinoma arising in micro glandular adenosis).

8. Patients with right to left cardiac shunts.
9. Pulmonary hypertension (defined as pulmonary arterial pressure greater than 25 mmHg).
10. Adult respiratory distress syndrome.
11. Uncontrolled hypertension (defined as persistent hypertension greater than 140/90 mmHg despite adherence to maximal doses of 3 antihypertensive agents one of which should be a diuretic)
12. Heart failure (defined as symptoms and/ or signs caused by a structural and/ or functional cardiac abnormality as determined by ejection fraction <50%, abnormal cardiac chamber enlargement, moderate/ severe ventricular hypertrophy or moderate/ severe valvular obstructive or regurgitative lesion and at least one of the following; elevated natriuretic peptide, objective evidence of cardiogenic pulmonary or systemic congestion by diagnostic modalities such as imaging or haemodynamic measurement at rest or with provocation).
13. Renal failure (defined as glomerular filtration rate <60 mL/min/1.73 m^2 for 3 months or more).
14. Recent thromboembolism (within 6 months of patient recruitment to study).
15. Hypercoagulation disorder.

1.3 Study design

A single-centre study to evaluate the use of high-resolution CEUS in TNBC tumours in patients undergoing NACT and correlate the imaging with established markers of angiogenesis, proliferation and vascular mimicry.

Patient recruitment and test procedures will be undertaken at Maidstone and Tunbridge Wells NHS Trust (MTW) in the Peggy Wood Breast unit and laboratory work at the University of Kent, School of Biosciences.

Sequential patients with newly diagnosed TNBC with planned NACT will be identified at the breast multi-disciplinary meeting (MDT) at MTW.

Eligible patients will be identified at the MDT by the direct clinical care team.

The study outline will be explained to the patients at their first outpatient appointment if they express an interest in being involved in the research, they will also be provided with a patient information leaflet (**Appendix 6.2**). Following this, they will attend an oncology appointment to plan NACT as well as a genetics clinic to have blood taken to look for germline mutations.

Patients agreeing to participate in the study will be given a unique trial number and be invited to attend the breast unit at MTW. They will then sign a consent form (**Appendix 6.1**) before beginning the study. The study outline is shown in **Figure 5**.

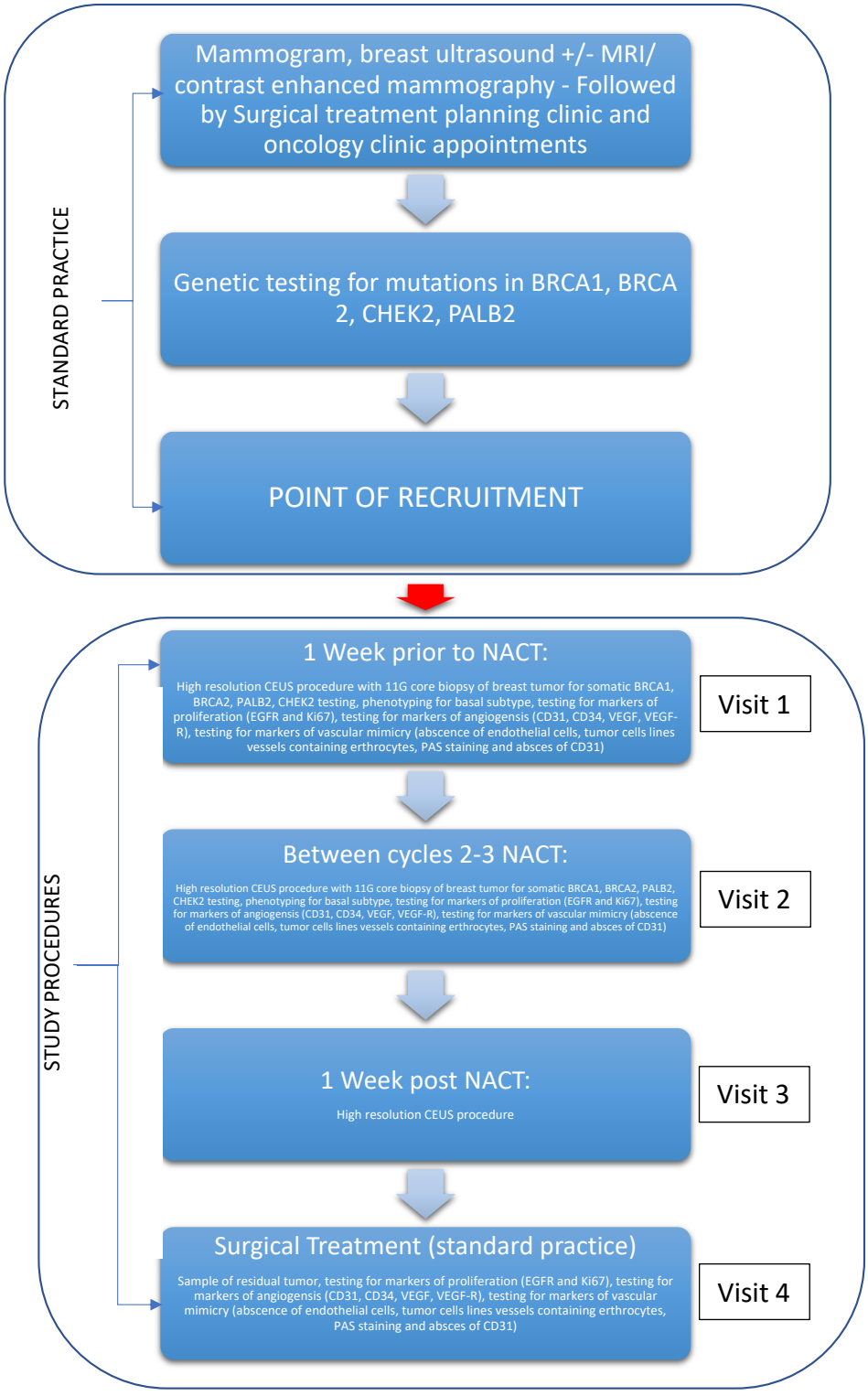


Figure 5 Study design. *Flow diagram of patient journey inclusive of standard NHS care and research participation. Standard NHS care consists of upfront imaging, confirmatory pathology, surgical planning and onward care via oncology with genetic testing prior to neoadjuvant chemotherapy (NACT). Research participation involves the above with the addition of high-resolution CEUS (HRCEUS) and biopsy before NACT, midway through NACT and HRCEUS post NACT with samples of residual tumour analysed from the final surgical specimen.*

At visit 1 the patient will have high-resolution CEUS breast imaging using the Vantage 256^{TM} Research platform (Verasonic Kirkland WA, USA) with a handheld transducer probe and 3 x 11g core biopsies of the tumour. This will be performed by a consultant radiologist at MTW. The images taken initially will be using existing ultrasound technology to identify the position of the tumour before progressing to the high-resolution CEUS. Images obtained will be stored for offline analysis to be undertaken by Professor Mengxing Tang's team at Imperial College London. The core biopsies will be transferred to MTW pathology department for processing and paraffin embedding before transfer to the School of Biosciences, University of Kent for analysis to look for markers of basal type phenotype using IHC (CK5/6, CK14), markers of proliferation (EGFR, KI67), markers of angiogenesis (CD34, CD31, VEGF, VEGF-R) and markers of vascular mimicry (H&E staining, PAS staining, absence of CD31)

At visit 2 (between cycles 2 &3 of NACT), patients will return for the same procedure as visit 1.

At visit 3 (end of NACT) patients will return for high-resolution CEUS and no further biopsies.

At visit 4 (post-NACT) samples will be taken from their definitive breast cancer surgery approximately 4 weeks after completion of NACT and processed by the MTW pathology department for IHC.

Chapter 2: Materials and methods (laboratory-based)

2.1 Cell line

The HCC38 triple-negative human breast cancer cell line was used to establish the IHC technique. This cell line was obtained from Professor Michaelis's lab but was not isolated by his group. It is epithelial in origin, isolated from breast tumour ductal tissue and is negative for the expression of the oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2). The tumour was classified as TMN stage IIb, Grade 3, with 3/28 lymph node metastasis (36). This cell line was utilised as it most closely represented the patient samples that would be obtained.

2.2 Cell Culture

HCC38 was cultured in Iscove's modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS). The cells were cultured at optimum conditions of 37°C and 5% CO_2 in a humidified incubator.

Initially, the cells were grown to confluence in a T25 flask before being transferred to a T75 flask. The cells were split by removing old media and discarding this from the flask and then washing the flask with 5 ml of phosphate-buffered saline (PBS). The cells were then detached from the flask using 1 ml Trypsin EDTA x10 solution and resuspended in fresh media. Approximately 1 ml of this new cell solution was transferred to a new T75 flask and resuspended in fresh media. Cells were split at an appropriate ratio (e.g., 1:10) every 3-5 days, depending on when they reached 75-85% confluence to ensure logarithmic phase growth for experimental procedures.

2.3 Cell Pelleting

Once the cells had reached an appropriate confluence (approximately 80%) and backup reserves had been stored for errors, cell pelleting was undertaken. A T75 flask which was 70% confluent was selected and media was removed discarded and washed with PBS. Following this, it was then trypsinised once more and resuspended in fresh media. The cell suspension was then centrifuged in a 15 ml falcon tube at 1200 rpm for 5 minutes. The supernatant media was then separated from the cell pellet and discarded. The cell pellet was then tapped to loosen it before further PBS was added to resuspend the pellet. This suspension was then centrifuged again and the process of decanting the supernatant and resuspending in PBS was repeated 3 times leaving only the cell pellet.

5 ml of fixative (70% ethanol) was added to the pellet and this cell suspension was vortexed mildly and fixed overnight at 4°C. This was then centrifuged at 1800 rpm for 10 minutes before decanting the supernatant and adding sequential increasing alcohol concentrations (70%, 90%,100%) over six hours with two hours of exposure at each concentration. The final stable cell pellet was removed from the tube and placed into a small beaker containing histogel (specimen processing gel -product code: 12006679 sold by Thermofisher Scientific) and left to incubate at room temperature for four hours.

This cell pellet and histogel combination was then transferred to a new beaker containing fresh histogel with careful transfer of the cell pellet. This was repeated 3 times in total. The cell pellet was then embedded into an immunohistochemistry (IHC) mould and allowed to cool at room temperature.

2.4 Cell Pellet Sectioning and Mounting

The prepared cell pellet was then sliced into sections of < 5 µm in thickness before being placed in a 37°C water bath. Each section was hand-mounted with the use of forceps onto charged glass slides ensuring appropriate adherence, and orientation. These slides were then cooled to room temperature before immunocytochemistry (ICC) being performed.

2.5 Immunocytochemistry

Immunocytochemistry (ICC) was performed on the HCC38 prepared slides initially. The initial protocol was optimised for appropriate conditions to ensure adequate staining. Parameters were altered including exposure time of each reagent during ICC and temperature controls. This protocol was used to stain all cell pellet sections. The final protocol is listed below:

Part 1

1. Slide/s of interest placed in glass staining dish and submerged in 100 ml of histoclear (xylene substitute as a clearing agent - National Diagnostics HS-200) for 4 minutes on laboratory rocker.
2. Slide/s transferred to new histoclear solution and process repeated
3. Slide/s transferred to new histoclear solution and process repeated.
4. Slide/s transferred to glass staining dish and submerged in 100 ml of 100% IMS for 4 minutes on laboratory rocker
5. Slide/s transferred to new glass staining dish containing 100% IMS and process repeated

6. Slide/s transferred to glass staining dish containing 70% IMS and process repeated
7. Slide/s placed in glass staining dish with distilled water for 5 minutes on laboratory rocker
8. Slide/s then examined and area of interest encircled with a hydrophobic pen to ensure ICC solutions remained on the area of interest.
9. Abcam Mouse-specific HRP/DAB (ABC) detection IHC kit (ab64259) then utilised. The initial step was to apply a hydrogen peroxide block to the area of interest and incubate at 4°C for 10 minutes.
10. Slide/s then resubmerged in fresh distilled water for 5 minutes
11. Slide/s placed in pre-mixed phosphate-buffered saline (PBS) solution for 4 minutes in a glass staining dish.
12. Slide/s transferred to new PBS solution and step 11 repeated.
13. Slide/s transferred to staining tray and protein block applied and left to incubate for one hour at 4°C.
14. Slide/s then placed in new PBS solution for 4 minutes
15. Slide/s then transferred to new PBS solution for 4 minutes
16. Slide/s then exposed to primary antibody of interest (**Table 1**) and left in covered staining tray for 24 hours at 4°C. Antibody dilutions were tested based on suppliers' recommendations.

Part 2:

1. Slide/s then placed in new PBS solution for 4 minutes
2. Slide/s then transferred to new PBS solution for 4 minutes

3. Secondary antibody (biotinylated goat anti-mouse) supplied with detection kit applied to the area of interest and incubated at 4°C for one hour.
4. Slide/s then placed in PBS solution for 4 minutes
5. Slide/s then transferred to new PBS solution for 4 minutes
6. Streptavidin peroxidase supplied with detection kit applied to the area of interest and left exposed for 20 minutes at 4°C
7. Slide/s then placed in PBS solution for 4 minutes
8. Slide/s then transferred to new PBS solution for 4 minutes
9. DAB chromagen mixed with DAB substrate and vortexed mildly for 2 minutes before being pipetted onto area of interest on slide
10. Colour change visualised under light microscopy and at first colour change slide submerged in distilled water to stop the reaction

2.6 Haematoxylin and Eosin staining

The final step of ICC and IHC involves counterstaining. This allows for visualisation of the anatomy. Haematoxylin stains nuclear components and eosin stains cytoplasmic components. This counterstaining step is essential to differentiate positive staining for the antibody of interest from background or improper staining. These steps were again optimised initially.

Optimised H&E staining:

1. Slide/s freshly stained with DAB chromagen then exposed to haematoxylin for 30 seconds before being washed under cold running water for 5 minutes
2. Slide/s then exposed to 1% eosin for 1 minute at room temperature
3. Slide/s then placed in sequential alcohol-containing glass staining dishes (70%, 100%, 100%) for 4 minutes each whilst on a laboratory rocker.

4. Slide/s then removed from the alcohol solution and the remaining alcohol was allowed to evaporate prior to dibutylphthalate polystyrene xylene (DPX) being applied via pipette and glass coverslip placed carefully to avoid air bubbles in the specimen.
5. Finally prepared slide/s were then visualised using an Olympus U-SDO™ microscope with a camera attachment and digital photographs taken at various magnifications (40x, 100x, 200x, 400x).

2.7 Clinical Study

As this study is a clinical study in human subjects' ethical approval was mandatory prior to delivering the study. The initial application for the study was processed via the integrated research application system (IRAS) with ethical approval granted to begin the study on 18/5/22 via the West of Scotland Research Ethics Service (WoSRES) (**Appendix 6.3**). Following this recruitment began and suitable patients were contacted and two eligible patients agreed to take part in the study. Following the completion of the research study, all tissue collected will be destroyed in compliance with the Human Tissue Act 2004 (**Appendix 6.5**)

2.8 Immunohistochemistry

IHC was performed on patient samples obtained at visits 1, 2 and 4 **(Figure 5)**. The same protocol was initially employed as that used for ICC of HCC38. The same antibodies were again tested at each stage (**Table 1**). This was then analysed in conjunction with a consultant pathologist based at Maidstone Hospital to evaluate the presence/absence of IHC staining with each antibody of interest.

Table 1 List of antibodies for ICC/ IHC. Antibody concentrations shown are those finalised for use by IHC.

Primary antibody	Supplier	Catalogue number	Species	Final dilution
Anti Ki67	Abcam	AB238020-1001	Mouse	1:100
Anti CD34	Abcam	AB54208-1001	Mouse	1:100
Anti VEGFA	Abcam	AB1316-1001	Mouse	1:100
Anti EGFR	Abcam	AB264540-1001	Mouse	1:100
Anti VEGFR	Abcam	Ab39378-1001	Mouse	1:100
Anti CD31	Abcam	AB9498-1001	Mouse	1:200
Anti CK 5	Abcam	AB17130-1001	Mouse	1:75
Anti CK6	Abcam	AB218438-1001	Mouse	1:500
Anti CK14	Abcam	AB7800-1001	Mouse	1:100

These antibodies were selected as they have been shown in previous studies to relate to mechanisms known to encourage cancer progression. Ki67 and EGFR were used as markers of proliferation, CD 34, VEGF, VEGF-R, and CD31 were selected as markers of angiogenesis and the absence of CD31, H&E and PAS staining to assess for a lack of endothelial cells as markers of vascular mimicry.

Chapter 3: Results

3.1 Optimisation of immunohistochemistry using the HCC38 triple negative human breast cancer cell line

The HCC38 cell line was maintained at a low density (**Figure 6**) until cell pelleting was required, at which point it was allowed to reach approximately 80% confluency and cell pelleting, fixation, embedding and sectioning were undertaken.

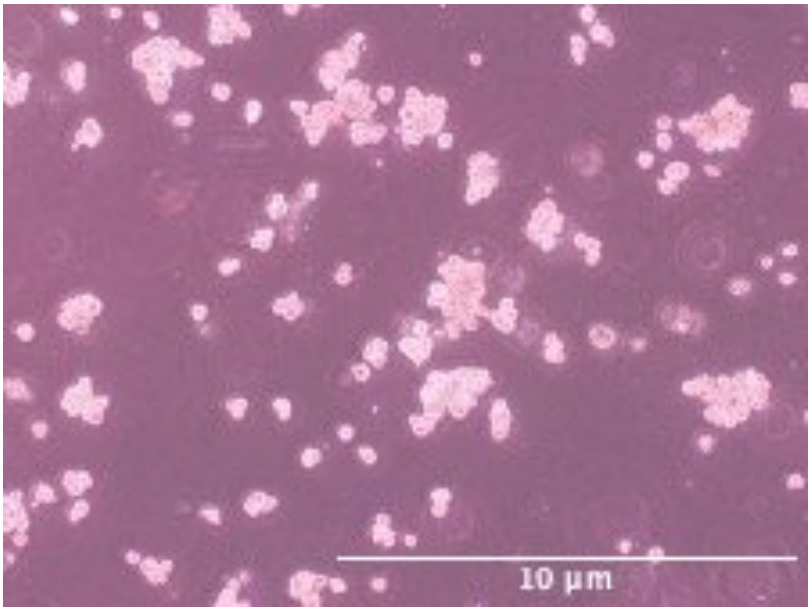


Figure 6 HCC38 triple negative human breast cancer cell line. *Cells were cultured then fixed and sections prepared and imaged according to the Materials and Methods. Image magnification at 100x captured at low density with Brightfield Microscope (Olympus U-SDO™)*

Following the preparation of slides ICC staining was undertaken in the form of haematoxylin staining initially with a standard compound microscope (Olympus U-SDO™) with camera attachment. As the images were of poor quality repeat images were undertaken with a compound microscope with a phone camera attachment to

improve the image quality (**Figure 7**). These images were able to clearly identify the individual cells in the HCC38 sample.

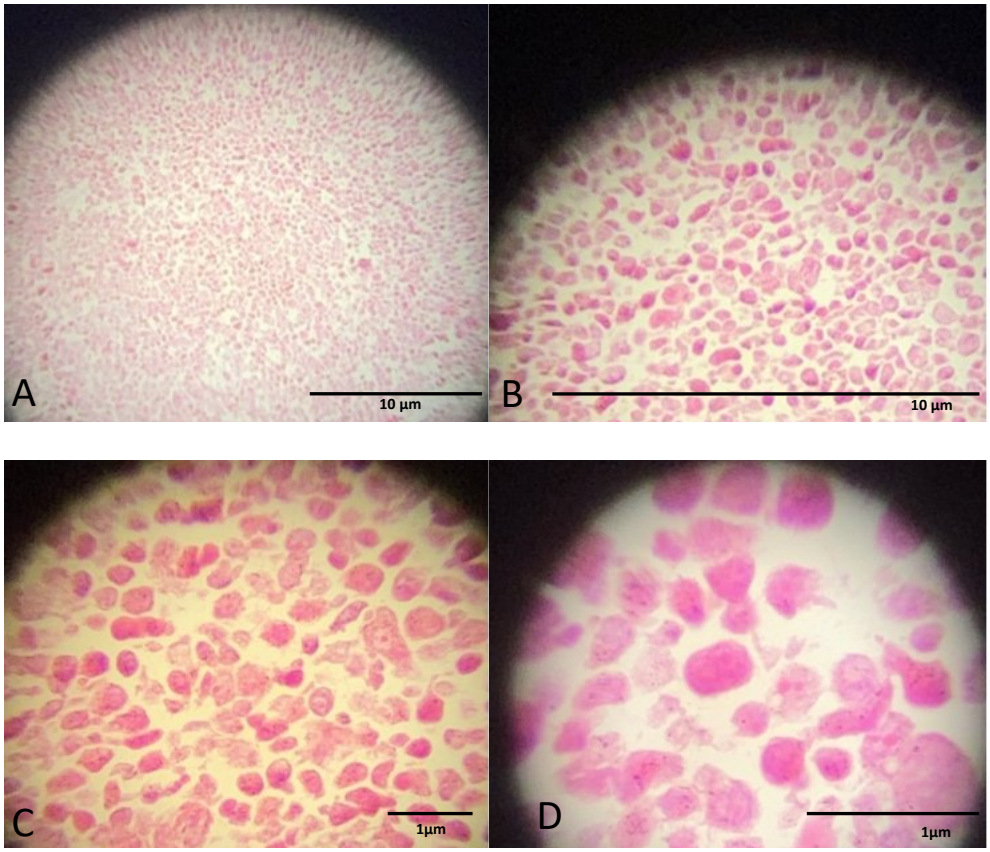


Figure 7 : Haematoxylin staining of the HCC38 triple negative human breast cancer cell line. *Cells were cultured and then fixed, stained with haemotoxylin, and sections prepared and imaged according to the Materials and Methods. Image magnifications are: (A) 40x, (B) 100x,(C) 200x,(D) 400x. Images captured using mobile phone camera through optical lens.*

Given this improvement in image quality, a dedicated camera (Swift) attachment was employed to improve the image quality and attached to the brightfield microscope to replace one eyepiece and allow digital representation of the slide using the inbuilt Olympus™ digital image capture software.

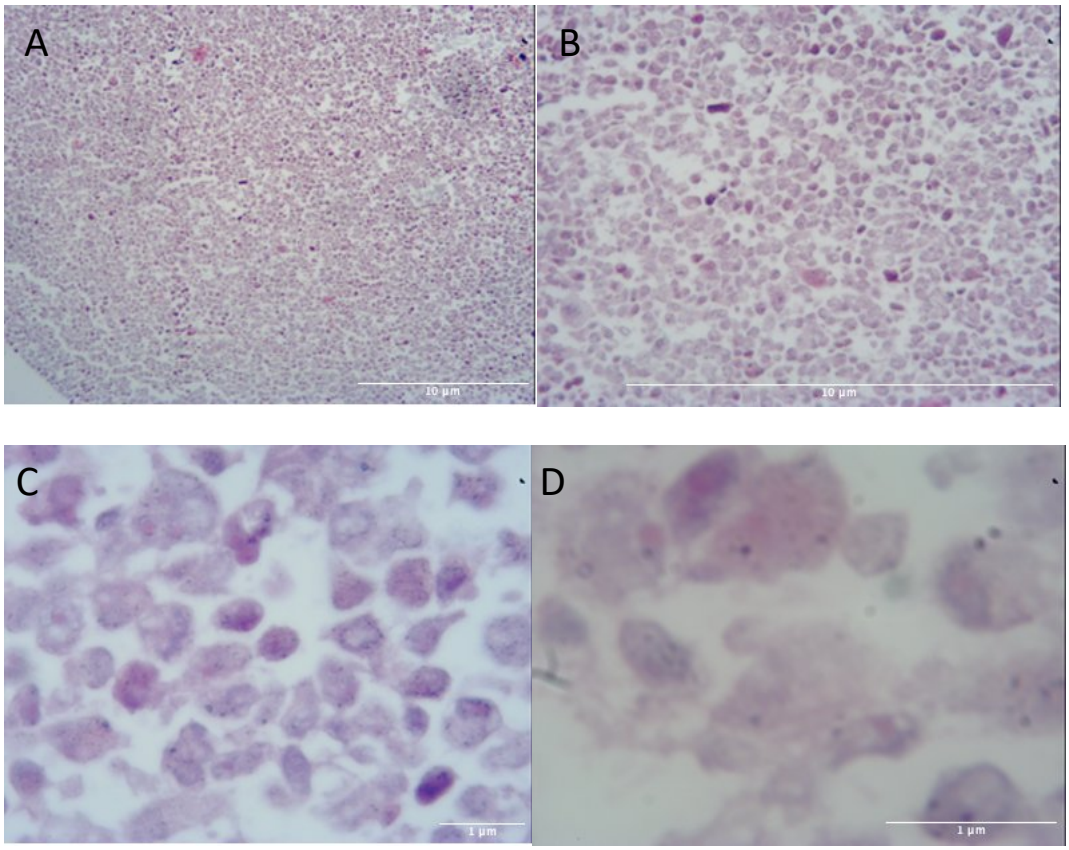


Figure 8 :Haematoxylin staining of the HCC38 triple negative human breast cancer cell line. *Cells were cultured and then fixed, stained with haemotoxylin, and sections prepared and imaged according to the Materials and Methods. Image magnifications are: (A) 40x, (B) 100x,(C) 200x,(D) 400x.*

Once image quality was satisfactory for the H&E staining (**Figure 8**) the process of ICC with Ki67 was attempted. Initial trials involved different antibody concentrations and different primary antibody incubation times to identify the correct incubation time and dilution ratios to minimise background staining and demonstrate antigen-specific staining. **Figure 9** demonstrates the early testing of HCC38 with Ki67 as the primary antibody. Detection of Ki67 is highlighted, as such the concentrations and timings for Ki67 were adjusted accordingly. Various timings and dilutions were trialled until an

appropriate protocol was produced which required primary antibody incubation for 24 hours at 4°C with varying antibody dilutions (**Table 1**).

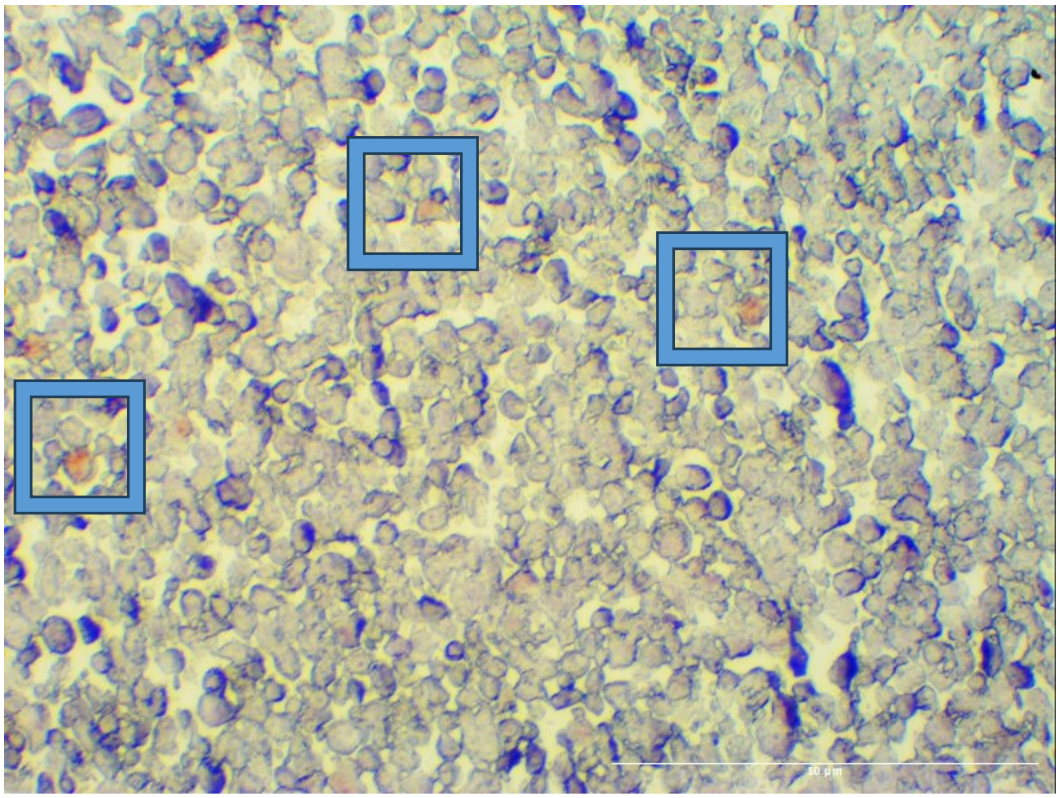


Figure 9: Ki67 Immunohistochemical staining of the HCC38 triple negative human breast cancer cell line. Cells were cultured and then fixed, sections prepared, stained to Ki67 antigen (1:75) according to the Materials and Methods. Image magnification of 100x with (highlighted areas demonstrating detection of primary antibody)

Once the protocol for antibody staining had been established, testing was undertaken on the remaining antibodies to establish appropriate primary antibody exposure and dilutions (**Table 1**). The initial results of ICC with the various primary antibodies are displayed in **Figure 10**.

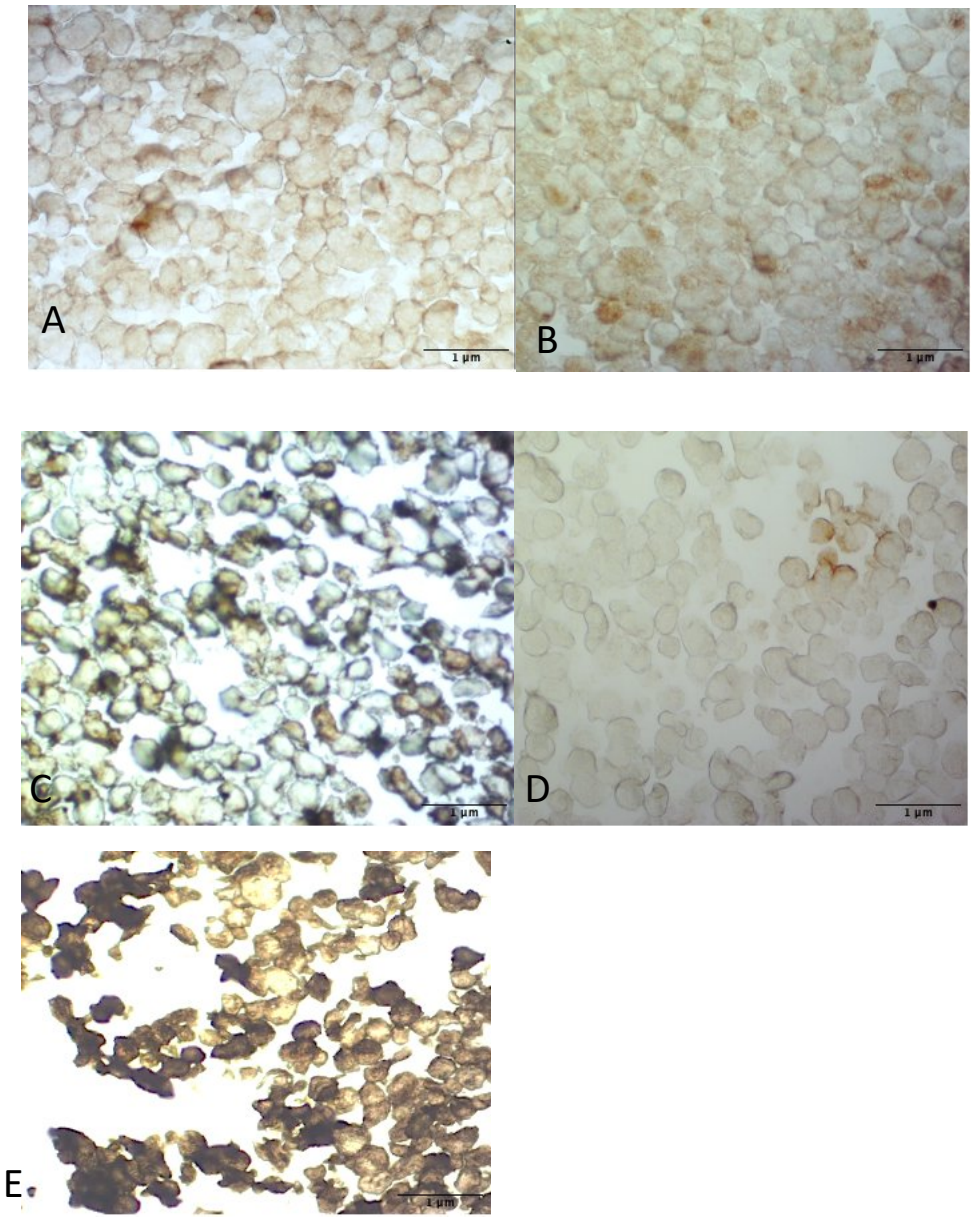


Figure 10 ***Immunohistochemical staining of the HCC38 triple negative human breast cancer cell line.*** *Cells were cultured and then fixed, sections prepared, stained with antibodies to specific antigens as indicated below, according to the Materials and Methods. Image magnification are x200 and primary antibodies and dilutions used (e.g. 1:100) as follows (A) EGFR 1:100, (B) VEGFA 1:100, (C) CD34 1:100, (D) CD31 1:200, (E) VEGFR 1:100.*

During the testing phase **(Figure 10)** whilst there was positive staining of appropriate chromagen location, there was a significant amount of background staining in many samples likely due to the initial IHC being undertaken on HCC38 which through cell pelleting does not retain tissue architecture. Given this, modifications were made to the protocol prior to testing on patient samples, inclusive of alterations to incubation times and antibody concentrations until the final protocol was established as detailed in the methodology and **Table 1**.

3.2 TNBC (testing)

Once appropriate antibody dilutions and incubation times had been established using sections from the HCC38 cell pellets, patient samples were trialled to see if these parameters were suitable for IHC as opposed to ICC. These samples were manually sectioned at the University of Kent using a microtome utilising blocks from the first patients' first visit (TNBC001) that were prepared at Maidstone and Tunbridge Wells NHS Trust (MTW). This avoided the need to use the sections provided by the pathology department at MTW.

Initially, haematoxylin and eosin (H&E) staining were undertaken (Figure **11**).

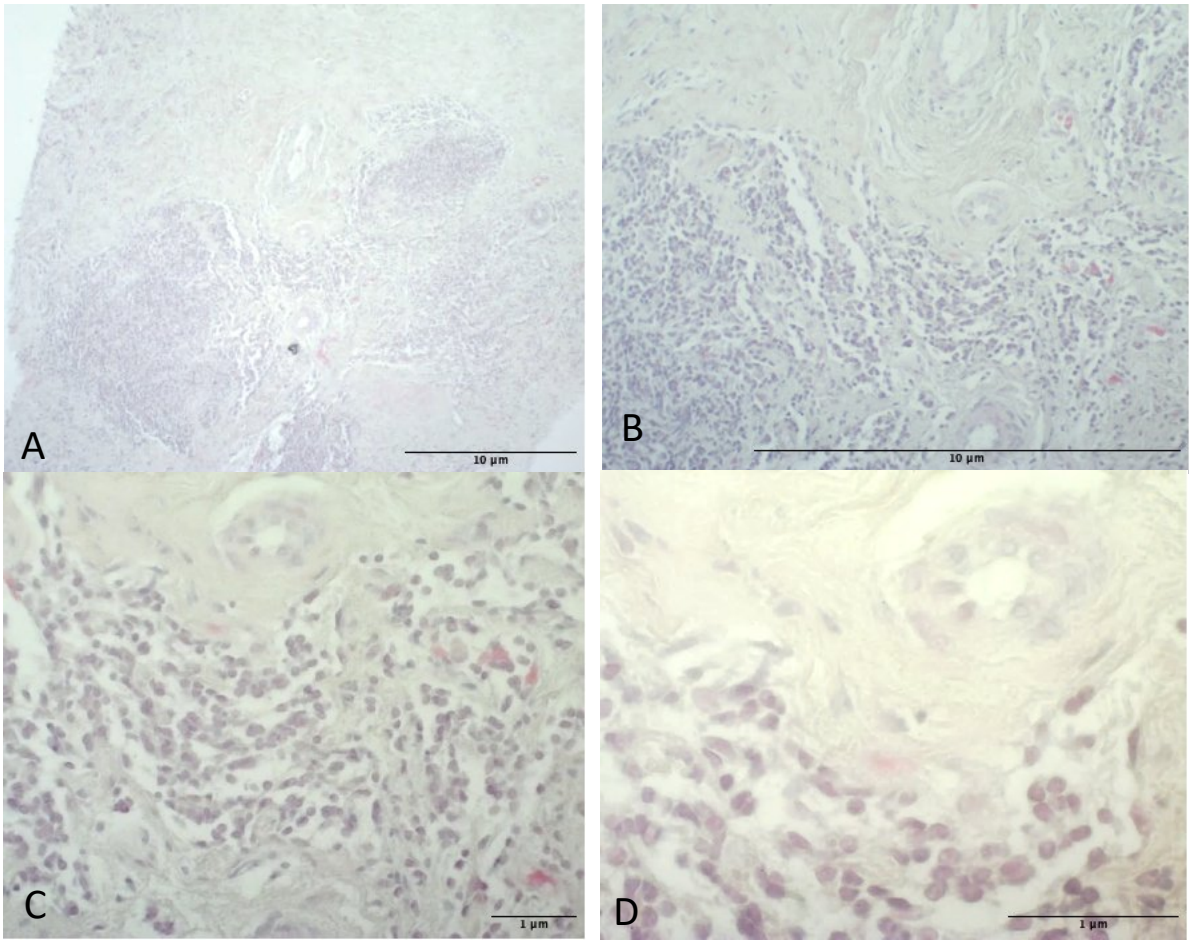


Figure 11 Immunohistochemical haemotoxylin and eosin staining of TNBC001. *Sections manually prepared with microtome to 5 µm and mounted on charged glass slides according to materials and methods. Image magnifications of* ***(A)*** *40x,* ***(B)*** *100x,* ***(C)*** *200x,* ***(D)*** *400****x.***

This H&E staining (Figure 11) demonstrated the specific tissue architecture from the core biopsy. At higher magnifications, a typical duct is seen with a single layer of myoepithelial cells and a single luminal layer. Surrounding this there were nests of cells with monomorphic nuclei appearing to invade the stroma indicative of an invasive carcinoma.

Antibody testing was then undertaken to establish appropriate dilutions and to assess if they were similar to those undertaken on HCC38 (**Figure 12**). Once again there was a large degree of background staining initially and this was found to be due to prolonged exposure time of the chromagen as the final step, this overexposure time was a matter of seconds in some cases. Given this, the chromagen was applied to the slide whilst under the microscope so that as soon as a colour change was evident the reaction could be halted in distilled water before counterstaining and mounting.

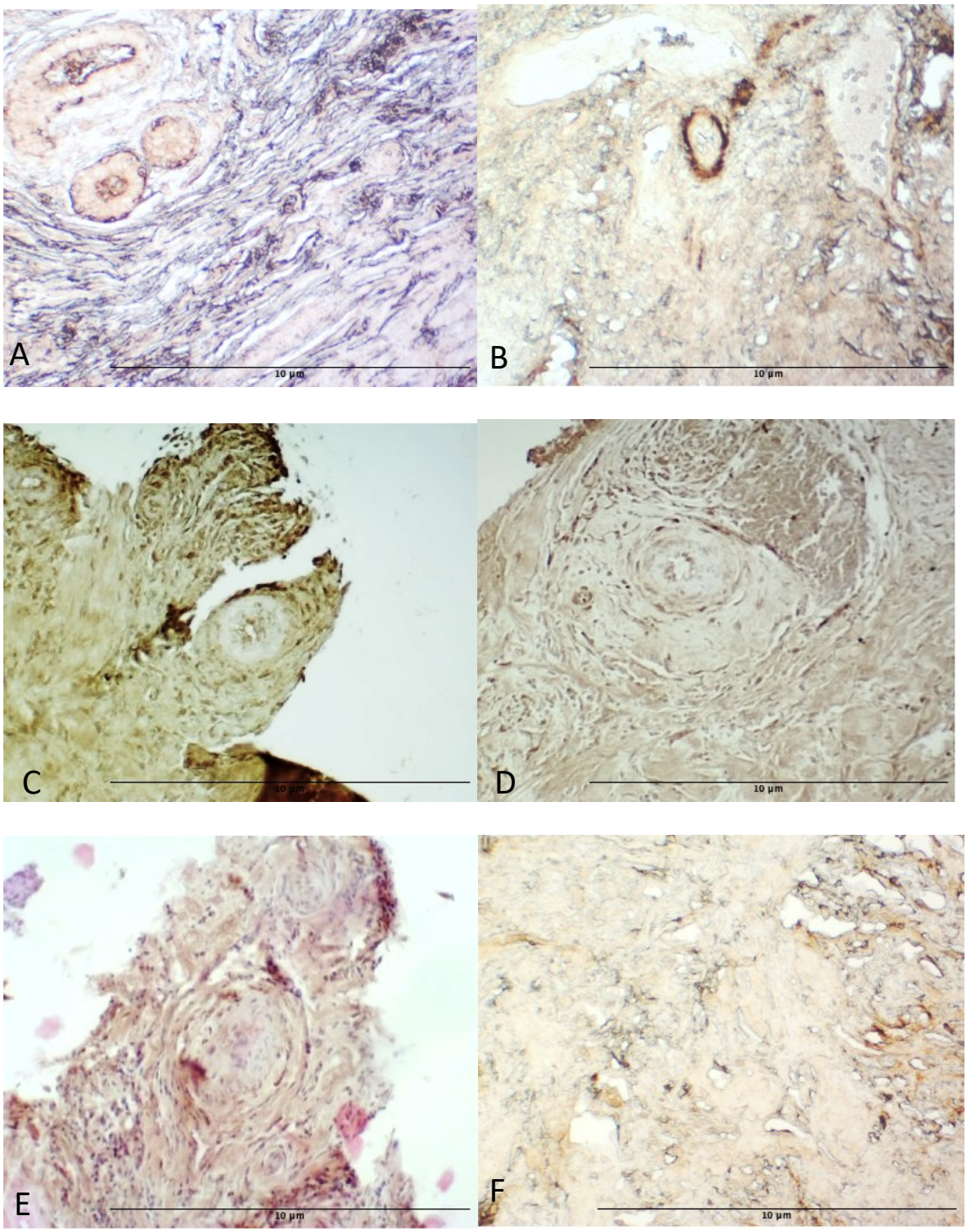


Figure 12 **Immunohistochemical staining of *TNBC001***. *Sections manually prepared with microtome to 5 µm and mounted on charged glass slides according to materials and methods. Image magnifications of 100x and primary antibodies and dilutions used (e.g. 1:100) as follows (A) VEGFR 1:100, (B) VEGFA 1:100, (C) CD34 1:100, (D) Ki67 1:100, (E) EGFR 1:100, (F) CD31 1:200*

The IHC images in Figure 12 represent final optimised dilutions for the various primary antibodies tested with appropriate staining of relevant antibodies.

3.3 Basal Antibodies

Following testing and ensuring the protocol was correct for markers of proliferation, angiogenesis and vascular mimicry, antibodies that were used to detect the basal type were tested (CK5, CK6, CK14 – **Figure 13**). This demonstrated that there was evidence of the basal type phenotype in the sample tested with appropriate staining. CK5, CK6, and CK14 primary antibodies used are located in the cytoplasm and were all detected in this sample and confirmed with negative control (absence of primary antibody).

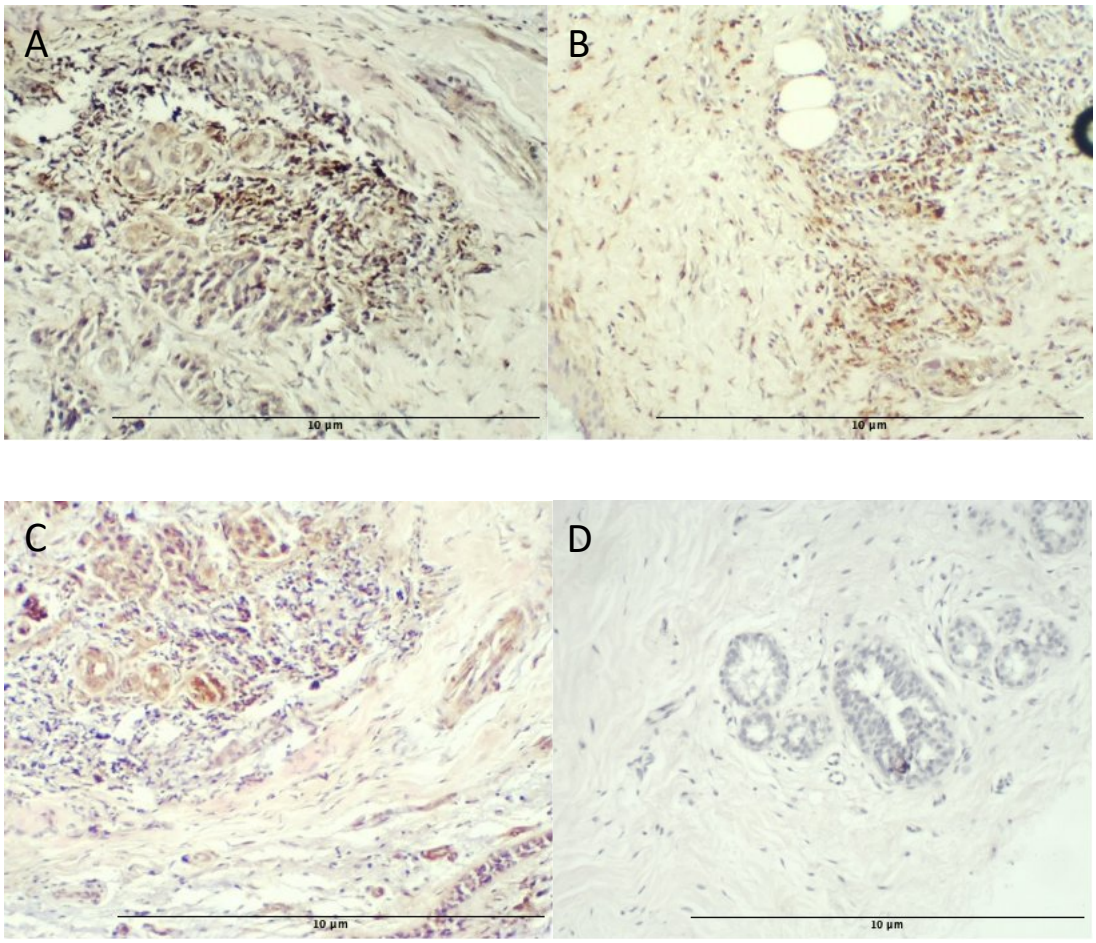


Figure 13 **Immunohistochemical staining of *TNBC001- basal antibodies.*** *Sections manually prepared with a microtome to 5 µm and mounted on charged glass slides according to materials and methods. Image magnifications of 100x and primary antibodies and dilutions used (e.g. 1:100) as follows (A) CK5 1:75, (B) CK6 1:500, (C) CK14 1:100, (D) Negative control (no primary antibody)*

3.4 Patient 1 (TNBC001)

TNBC001 initially presented to the breast department at MTW on 02/11/2022 and baseline standard imaging was undertaken (**Figure 14**). Standard imaging undertaken on first presentation to a breast clinic consists of bilateral breast mammogram (in those 40 years or above) utilising a two-view approach (craniocaudal CC and medial-lateral oblique MLO views) followed by a targeted standard breast B mode ultrasound for any areas of concern that were either detected clinically or mammographically. Initial imaging demonstrated concordance with clinical findings of a small mobile palpable lump in the left upper outer quadrant which on mammogram was found to be 19.6mm in maximal dimension and a further distinct area noted 9mm in maximal size. Ultrasound confirmed these lesions were at the 3 o'clock position (TNBC001A) and 12 o'clock position (TNBC001B) in the breast.

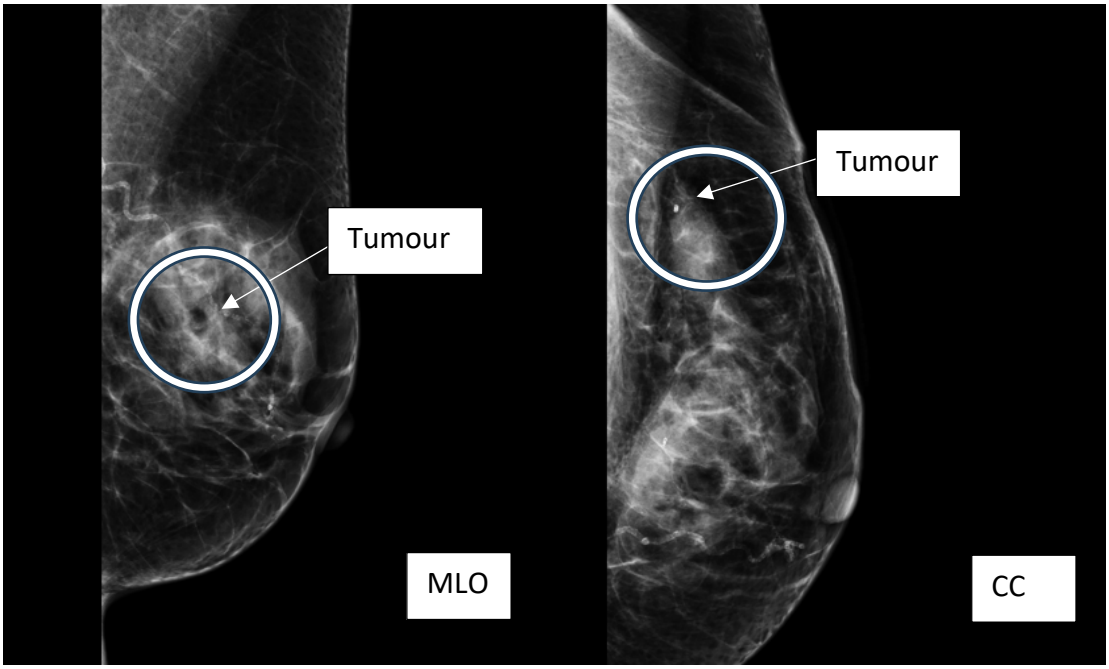


Figure 14 Mammography TNBC001 left breast. *Standard two view representation (Left image medial lateral oblique view (MLO), Right image craniocaudal view (CC). Areas highlighted represent detected area of suspicion.*

A core biopsy was undertaken and titanium marker clips were left at the sites of biopsy. Following this magnetic resonance imaging (MRI) was advised due to the

multifocal nature of the disease which is the current gold standard practice. This MRI demonstrated no other areas of disease but did demonstrate that TNBC001A and TNBC001B had central areas of necrosis (Figure 15) Core biopsies returned identifying both areas as grade 3 invasive ductal adenocarcinomas which were ER, PR and HER2 negative (representing a TNBC) and she was discussed at the breast multi-disciplinary meeting (MDT) with a plan made for further imaging and NACT.

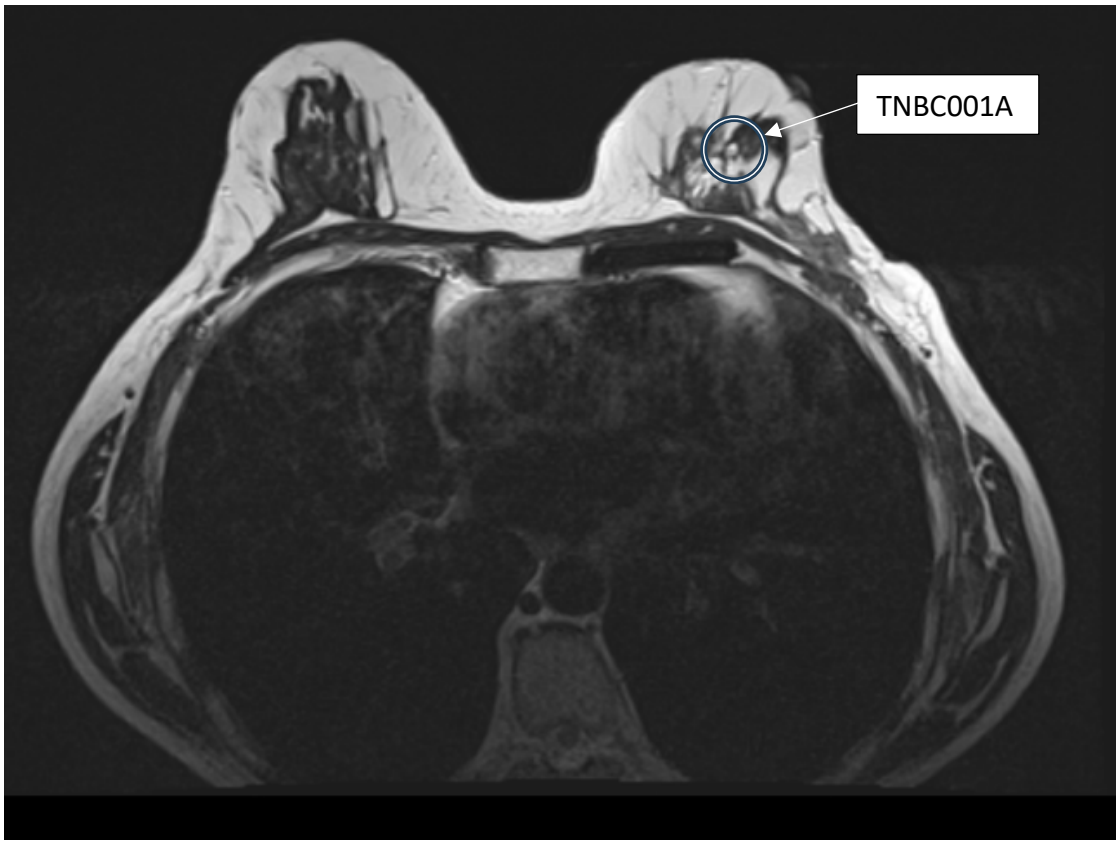


Figure 15 MRI with contrast both breasts. *Left breast with central necrosis in TNBC001A (identified by highlighted area (TNBC001B not visualised at this level on MRI image).*

As the multifocal nature of the disease was confirmed, TNBC001 also went on to have a positron emission tomography flurodeoxyglucose (PET FDG) scan to exclude distant disease. Again, thankfully no other distant disease was noted (**Figure 16**). This PET FDG image demonstrates the presence of an area of high metabolic activity as demonstrated by the highlighted area. No other areas were found abnormally to represent high metabolic activity apart from the known primary breast cancers already identified.

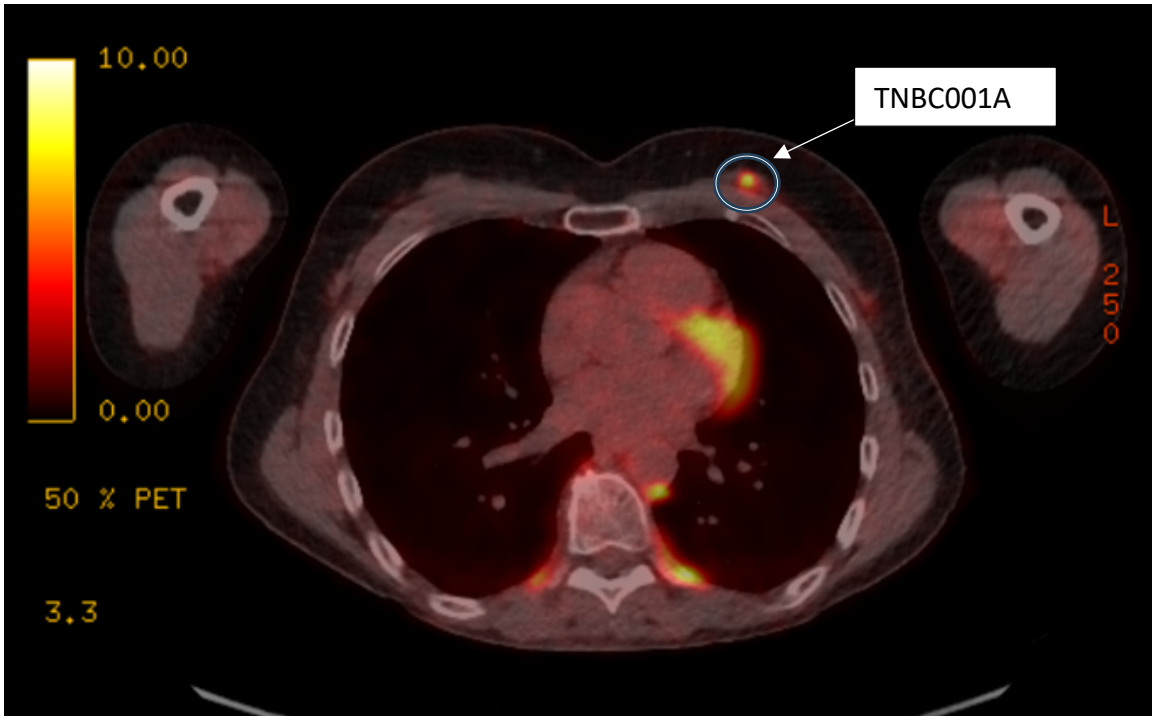


Figure 16 PET FDG imaging of TNBC001 *(TNBC001A in left breast only seen in this image slice demonstrated by highlighted area as highly metabolically active area)*

Once all imaging had been undertaken and prior to the patient commencing NACT she was approached to take part in the research study. The timeline of research participation is detailed in Table 2.

Table 2 – Timeline of TNBC001

Visit number:	Visit Date	Intervention
1	05/12/22	Imaging and core biopsy
2	16/1/23	Imaging and core biopsy
3	03/4/23	Imaging alone
4	18/4/23	Surgery

Following Visit 1 the patient was referred to the medical oncologists. She was found to be negative for germline mutations and was started on a NACT regimen consisting of epirubicin, cyclophosphamide and paclitaxel for 8 cycles. This NACT regime was completed prior to visit 3 and she additionally had a post-treatment MRI which demonstrated a radiological partial response to treatment. TNBC001 went on to have surgery in the form of a left mastectomy with planned delayed breast reconstruction. The final histology from surgery demonstrated a complete pathological response (the absence of residual invasive/ in situ cancer on the complete resected specimen) to NACT.

3.4.1 Immunohistochemistry

TNBC001 completed the research study and at visit 1, 2, 4 tissue samples were taken. Each was processed by MTW pathology department prior to IHC being performed (**Figures 17, 18, 19, 20**) to test for markers of proliferation (Ki67, EGFR), angiogenesis (VEGFR, VEGFA, CD34, CD31) and vascular mimicry (CD31 absence, PAS staining). Simultaneously, positive controls were assessed using HCC38 cell line samples for all the antigens of interest as this cell line was known to express the markers to be investigated (Appendix 6.6) whilst negative controls (no primary antibody) were assessed initially on the HCC38 cell line as well as on patient samples (Figure 18).

3.4.1.1 TNBC001A

The IHC performed on TNBC001A during the visits demonstrated clear differences between the amount of staining seen on progressive visits in many of the antibodies tested (**Figure 17**). H&E controls (negative controls) were additionally undertaken and displayed in Figure 18.

Specifically:

Ki67 – In the slides stained at each visit there was no clear staining of relevant areas to indicate the presence of Ki67 within the nucleus.

EGFR – Was found to show a decreasing appearance during the subsequent visits with only a small amount noted at visit 3. Therefore, showing a reduction in the amount of EGFR expressed.

VEGFR – Initial small degree of staining on visit 1 with more evidence of staining on visit 2 and a reduction in staining at visit 3.

VEGFA – High degree of positive and background staining at visit 1 with subsequent reductions in the levels of staining at visits 2 and 3.

CD34 – High degree of background staining and positive staining at visits 1 and 2 with a clear reduction in the amount of staining produced at visit 3.

CD31 – Present at Visit 1 and decreased presence at subsequent visits.

Basal antibodies tested in TNBC001A were present in all of the samples at Visit 1. No further visits were tested (**Figure 20**). This indicates that TNBC001A likely phenotypically represents a basal type subtype of TNBC as evidenced by the presence of CK5, CK6 and CK14 (37).

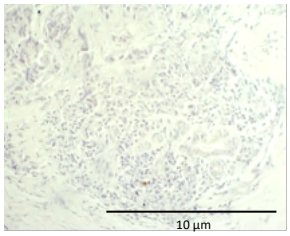
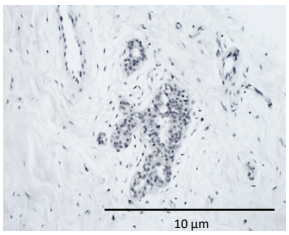
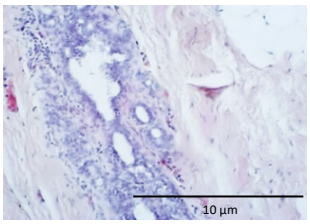
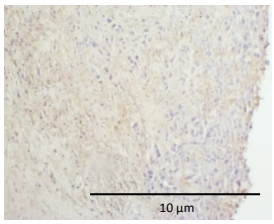
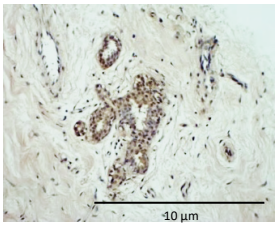
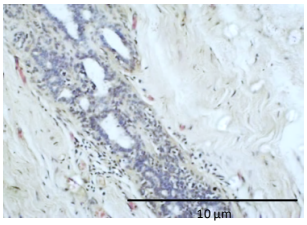
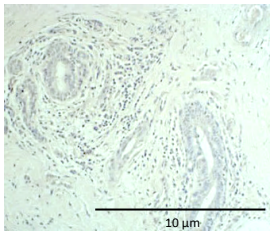
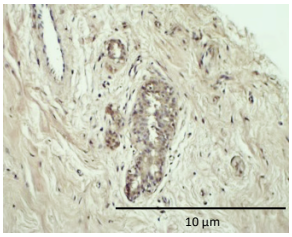
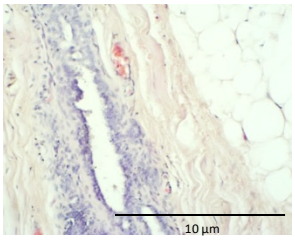
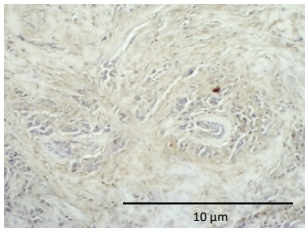
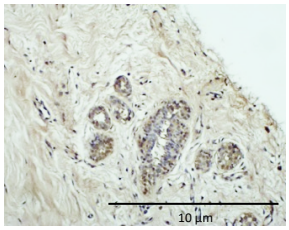
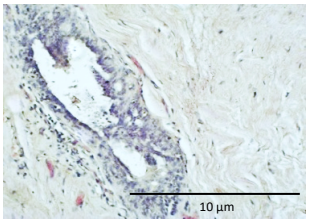
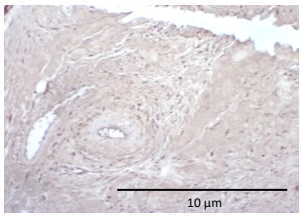
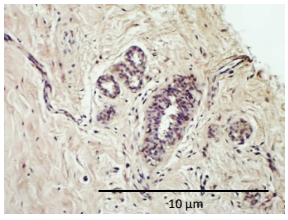
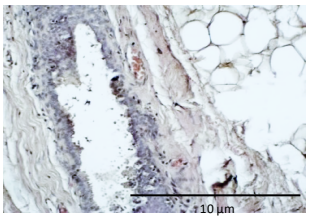
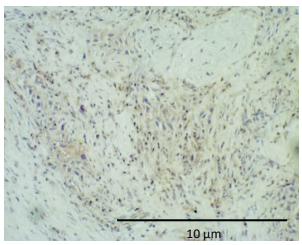
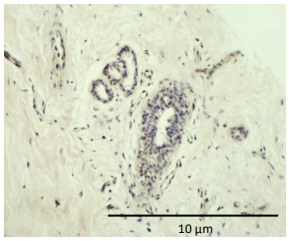
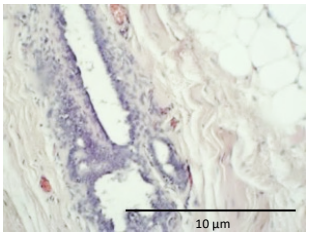
Antibody, dilutions and magnification	Visit 1	Visit 2	Visit 4
Ki67 1:100 100			
EGFR 1:100 100x			
VEGFR 1:100 100x			
VEGFA 1:100 100x			
CD34 1:100 100x			
CD31 1:200 100x			

Figure 17 – TNBC 001A – Immunohistochemistry staining of markers of proliferation (EGFR, Ki67), markers of angiogenesis (CD34, CD31, VEGF, VEGF-R) and vascular mimicry (PAS, CD31 absence). *Dilutions of each marker and magnification in figure. All visualised as per materials and methods.*

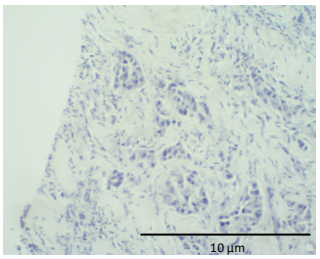
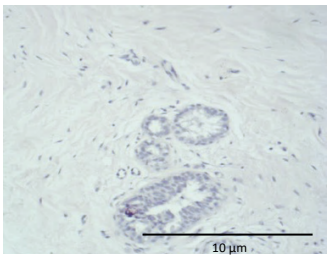
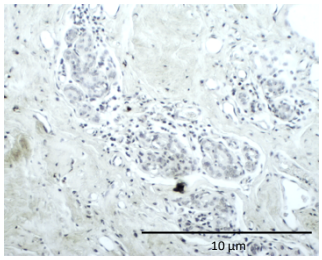
Antibody, dilution and magnification	Visit 1	Visit 2	Visit 4
TNBC001A No Primary antibody 100x			

Figure 18 – TNBC001A – Immunohistochemistry with no primary antibody (negative control) with H&E counterstaining. *No evidence of improper staining. All visualised as per materials and methods..*

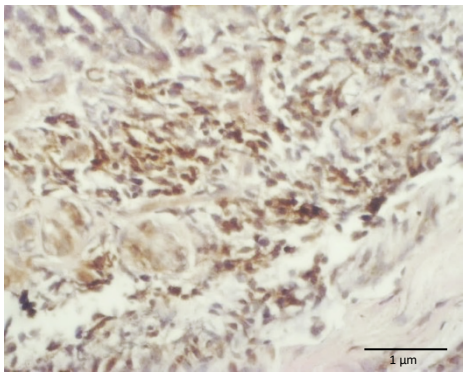
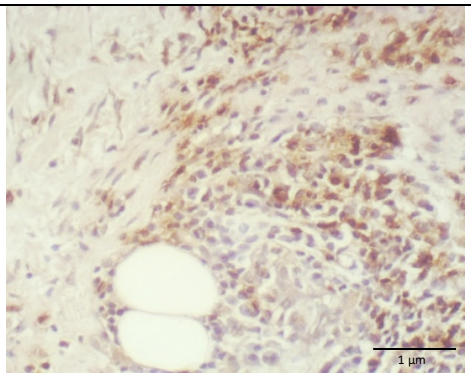
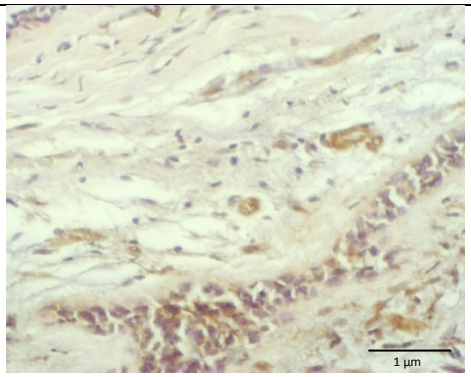
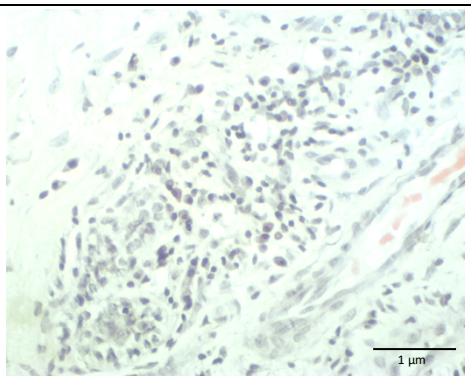
Antibody	Visit 1
CK5 1:75 200x	
Ck6 1:500 200x	
Ck14 1:100 200x	
No primary antibody (negative control) 200x	

Figure 19 – TNBC001A – basal antibodies (CK5, CK6, CK14). *IHC tested at Visit 1 to assess for phenotype in this sampled area and negative control with no primary antibody. All visualised as per materials and methods.*

There was no evidence of positive PAS staining in TNBC001A with endothelial cells being visualised throughout each visit and therefore no signs of vascular mimicry (**Figure 20**).

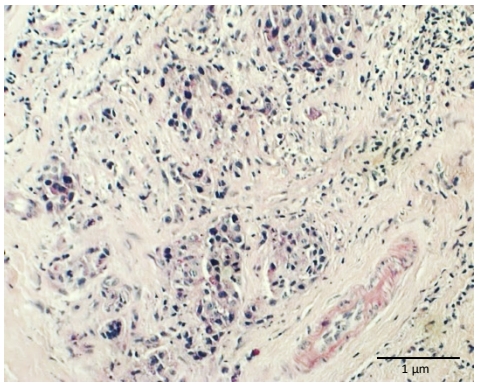
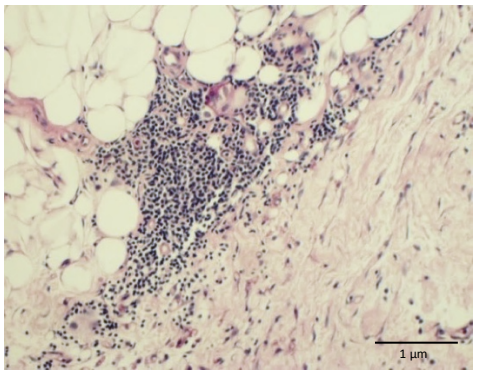
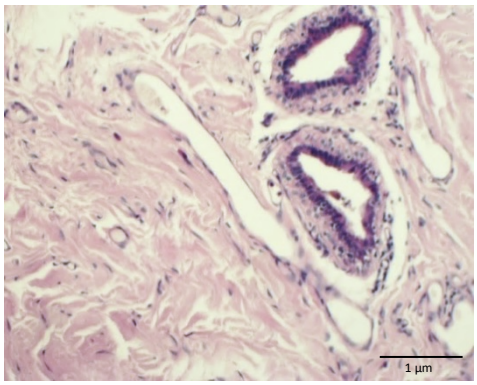
TNBC001 A	PAS staining 1:100
Visit 1 200x	
Visit 2 200x	
Visit 4 200x	

Figure 20 – TNBC001A PAS staining undertaken at each visit to assess for the presence of vascular mimicry (VM). *No VM demonstrated in this sampled area. All visualised as per materials and methods.*

3.4.1.2 TNBC001B

The IHC performed on TNBC001B during the visits demonstrated clear differences between the amount of staining seen on progressive visits in many of the antibodies tested which is more profound than that seen in TNBC001A (**Figure 21**). Additionally, negative controls were undertaken with the removal of the primary antibody (Figure 22) demonstrating no evidence of improper staining.

Ki67 – At visit 1 there was clear staining of multiple nuclei in the area of interest. This was continued to a lesser extent at visit 2 whilst at visit 3 no detectable staining was noted. Thus, demonstrating the presence of Ki67 in this area.

EGFR – Present at visit 1 in multiple areas which persisted at visit 2 and was minimally present at visit 3.

VEGFR – Clear staining visible at visit 1 which decreased on subsequent visits.

VEGFA – High degree of positive staining at visit 1 and minimal to no staining seen at visit 2 and 3.

CD34 – High degree of staining throughout visit 1 and 2 which decreased but still present at visit 3.

CD31 – Small amount present at visit 1, more evident at visit 2 and none visible at visit 3.

Basal antibodies tested in TNBC001B were found to be positive for CK5, CK6 but not for CK14 (**Figure 23**). This indicates this area most likely phenotypically represents a basal subtype but is not as evident as TNBC001A. Given the fact that TNBC001A was found to be of the basal type of TNBC it is more likely that TNBC001B represents the same type phenotypically

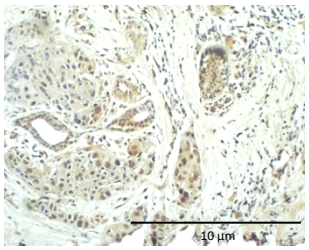
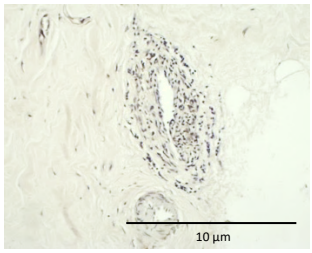
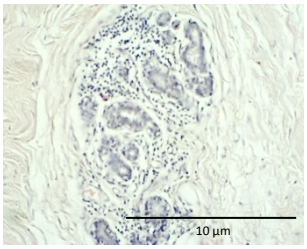
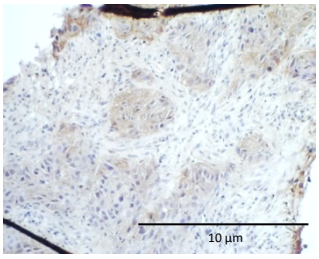
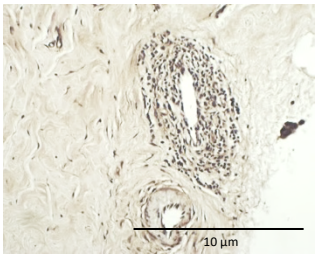
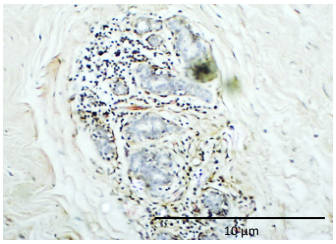
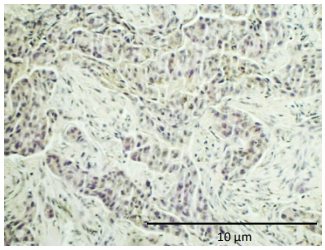
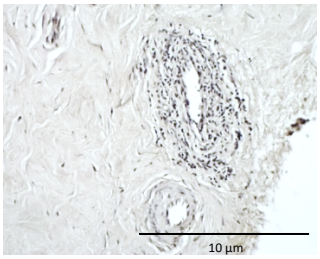
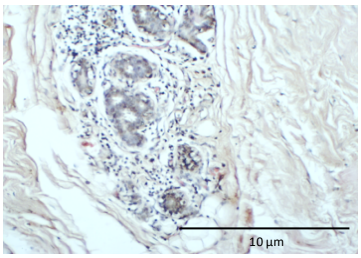
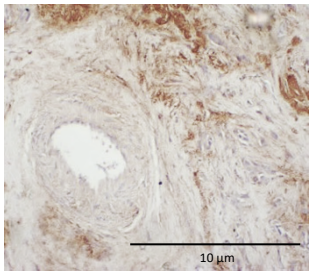
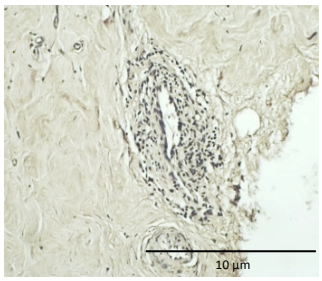
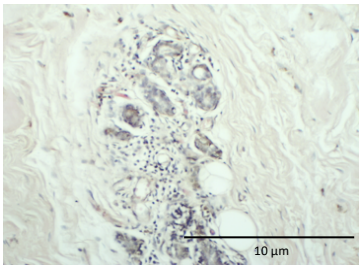
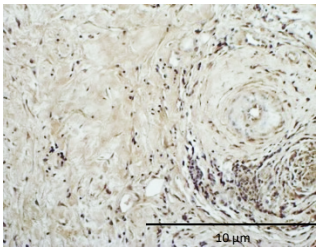
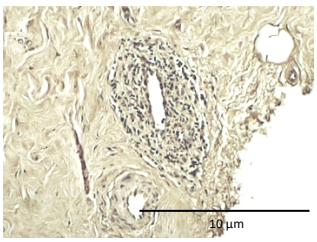
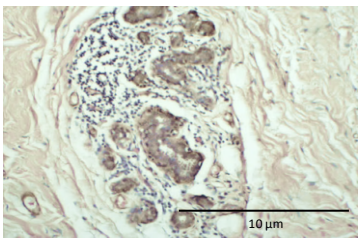
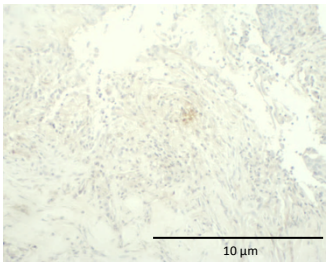
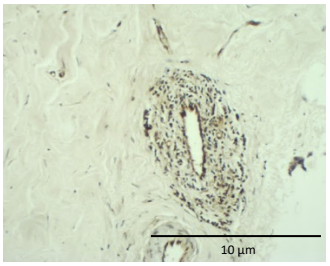
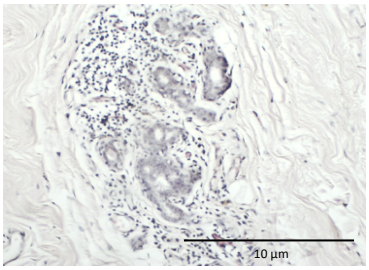
Antibody	Visit 1	Visit 2	Visit 4
Ki67 1:100 100x			
EGFR 1:100 100x			
VEGFR 1:100 100x			
VEGFA 1:100 100x			
CD34 1:100 100x			
CD31 1:200 100x			

Figure 21 – TNBC001B– Immunohistochemistry staining of markers of proliferation (EGFR, Ki67), markers of angiogenesis (CD34, CD31, VEGF, VEGF-R) and vascular mimicry. *Dilutions of each marker and magnification in figure. All visualised as per materials and methods.*

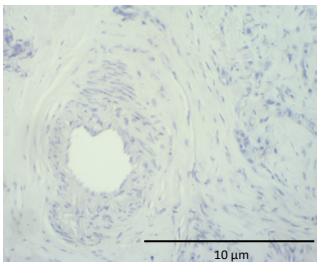
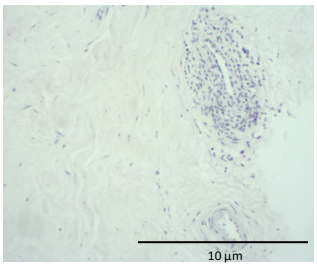
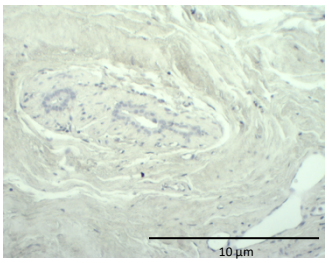
Antibody, dilution and magnification	Visit 1	Visit 2	Visit 4
TNBC001B No Primary antibody 100x			

Figure 22 – TNBC001A – Immunohistochemistry with no primary antibody (negative control) with H&E counterstaining. *No evidence of improper staining. All visualised as per materials and methods.*

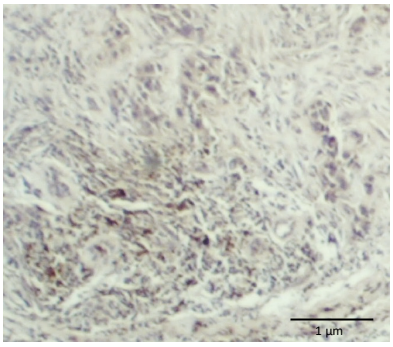
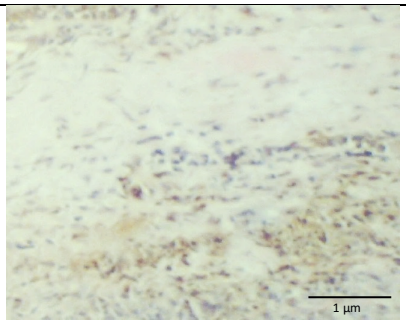
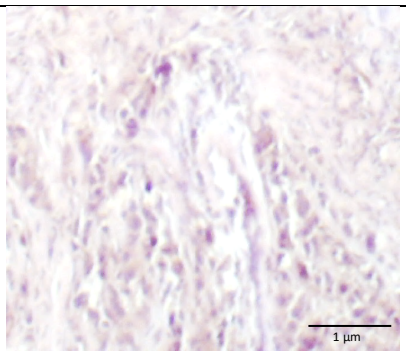
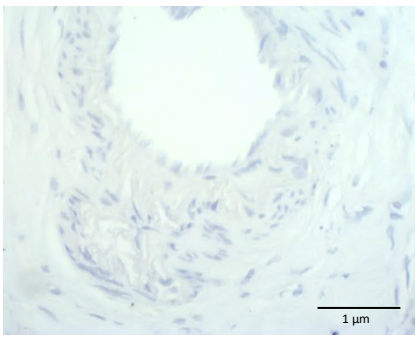
Antibody	Visit 1
CK5 1:75 200x	
Ck6 1:500 200x	
Ck14 1:100 200x	
No primary antibody (negative control) 200x	

Figure 23 TNBC001B – basal antibodies (CK5, CK6, CK14). *IHC tested at Visit 1 to assess for phenotype in this sampled area and negative control with no primary antibody. Demonstrating evidence of basal type in this sample. All visualised as per materials and methods..*

Pas staining undertaken for TNBC001B at all visits did not demonstrate any evidence of loss of endothelial cells and there were no signs of positive PAS staining in this area throughout the study and therefore no indications of vascular mimicry (**Figure 24**).

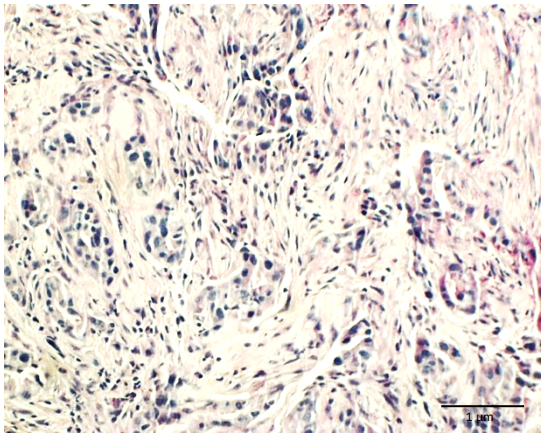
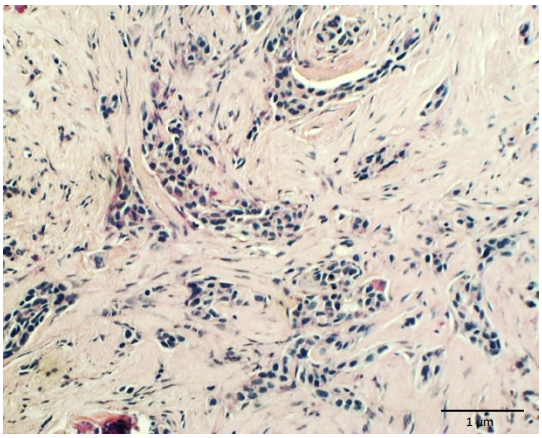
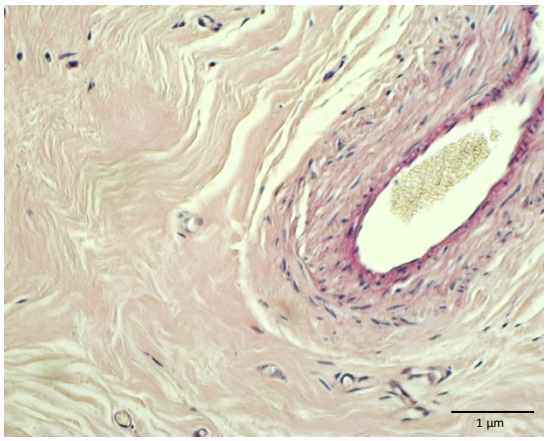
TNBC001 B	PAS staining 1:100
Visit 1 200x	
Visit 2 200x	
Visit 3 200x	

Figure 24 TNBC001B PAS staining undertaken at each visit to assess for the presence of vascular mimicry (VM). *No VM demonstrated in this sampled area. All visualised as per materials and methods.*

3.4.2 Imaging

Initially, TNBC001A and TNBC001B were identified through the use of standard NHS B mode ultrasound and performed by a consultant radiologist at MTW. Once the consultant radiologist was satisfied with the position of the probe this was switched to the Vantage 256^{TM} Research platform and high-resolution CEUS was performed. A bolus intravenous injection of microbubbles was infiltrated and these were tracked to the area of interest. Multiple images were undertaken at 90˚ and 180˚ utilising contrast-enhanced imaging at the time. Post-processing analysis of the obtained images was undertaken by Professor Mengxing Tang's team at Imperial College London. Initially, segmentation of the data was performed to isolate the region of interest (ROI). This was then followed by the identification of the microbubbles and digital subtraction of irrelevant points to leave only the super localisation of the microbubbles (**Figures 25 and 26**).

For TNBC001A (**Figure 25**) we can see that the same area was identified clearly at subsequent visits. The region of interest (ROI) at each visit decreased in its appearance, indicating a reduction in the size of the tumour at this site. This area also demonstrated a central area of opacity which when correlated with initial NHS standard imaging demonstrated this area represented a central area of necrosis. The results from the contrast-enhanced ultrasound (CEUS) also demonstrated that there was a great degree of vascularity around this tumour however much of this was distant from the ROI indicating a likely rapidly growing tumour which may have outgrown its blood supply. Despite this on subsequent visits we can see a reduction in the vasculature in CEUS which is evident when the super localisation is observed around the ROI.

3.4.2.1 TNBC001A

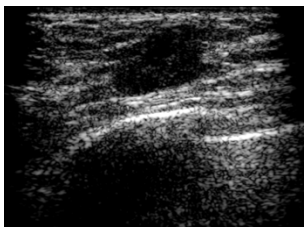
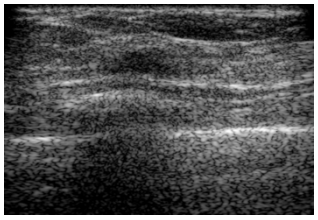
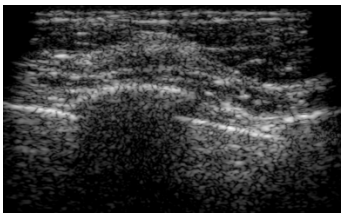
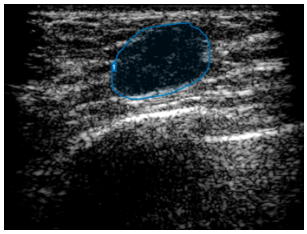
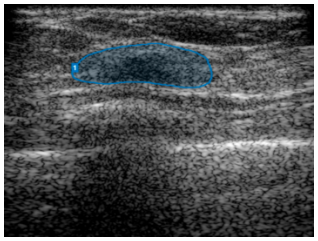
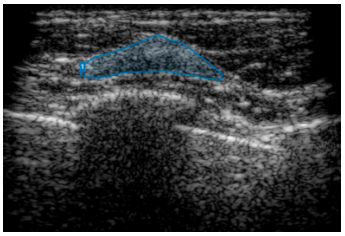
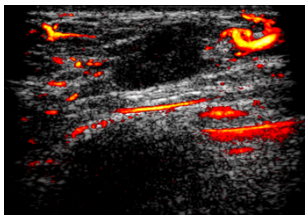
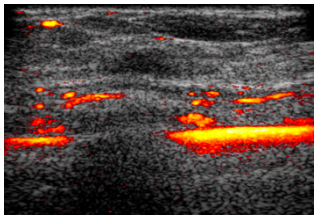
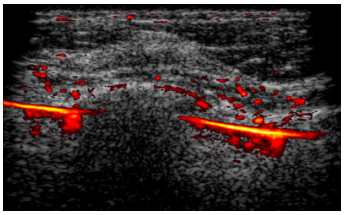
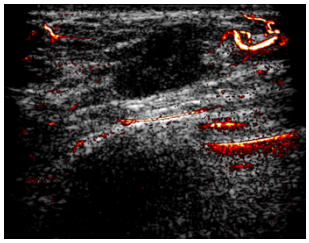
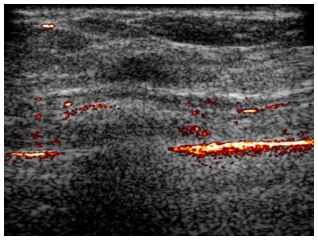
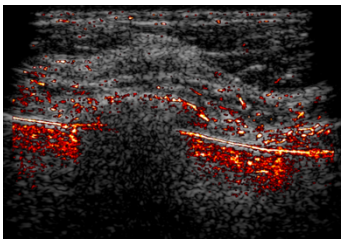
Imaging modality	Visit 1	Visit 2	Visit 3
Standard B mode ultrasound			
Standard B mode ultrasound with ROI			
Contrast enhanced ultrasound (CEUS)			
Super localisation of microbubbles			

Figure 25 – Imaging TNBC001A area. *Standard B mode ultrasound with and without highlighted region of interest. CEUS area of whole field of view with further super localisation of microbubbles around region of interest which appears to reduce during subsequent visits.*

3.4.2.2 TNBC001B

TNBC001B demonstrated a similar appearance to TNBC001A where there was a reduction in the ROI and a decrease in the vasculature surrounding this which was more pronounced on super localisation images (Figure 26). TNBC001 completed the research study in its entirety. At the time that she was recruited, immunotherapy had not been widely implemented as standard of care for TNBC patients. As such TNBC001 received NACT only prior to surgical intervention. Nevertheless, both standard imaging and pathology findings demonstrated that the patient had no radiological evidence or pathological evidence of remaining disease in either TNBC001A or TNBC001B.

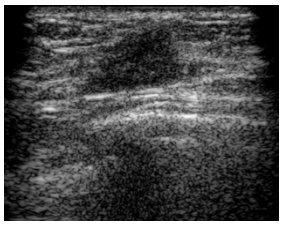
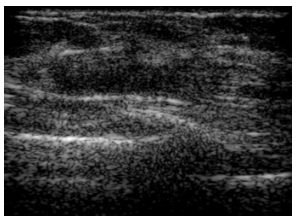
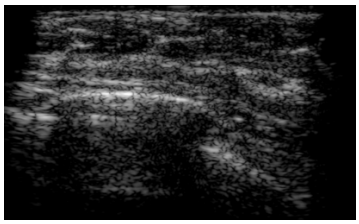
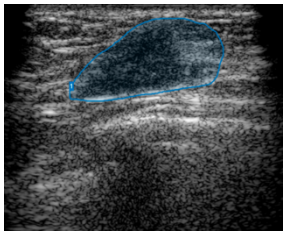
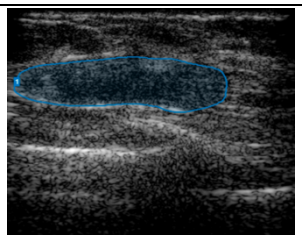
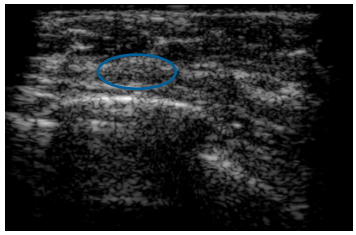
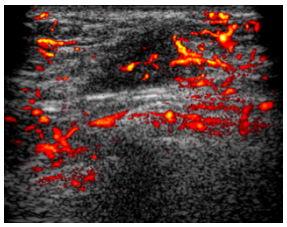
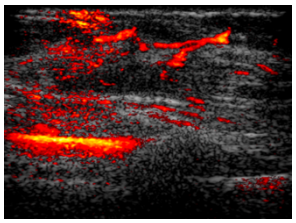
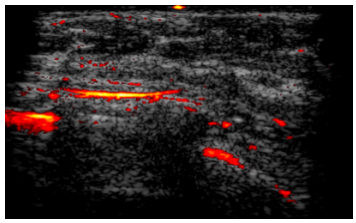
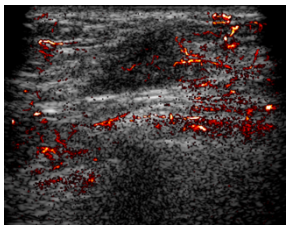
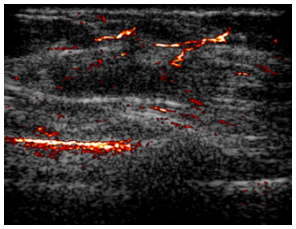
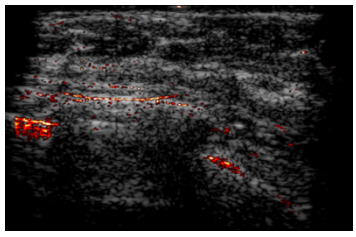
Imaging modality	Visit 1	Visit 2	Visit 3
Standard B mode ultrasound			
Standard B mode ultrasound with ROI			
Contrast enhanced ultrasound			
Super localisation of microbubbles			

Figure 26 – Imaging TNBC001B area*. Standard B mode ultrasound with and without highlighted region of interest. CEUS area of whole field of view with further super localisation of microbubbles around region of interest which appears to reduce during subsequent visits.*

3.5 Patient 2 (TNBC002)

TNBC002 presented to the breast department at MTW on 8/2/23 and clinical examination and NHS standard imaging was performed (mammography and B mode ultrasound) (**Figure 27**). This demonstrated two areas of suspicion in the left breast which were subject to a core biopsy and a marker clip placement.

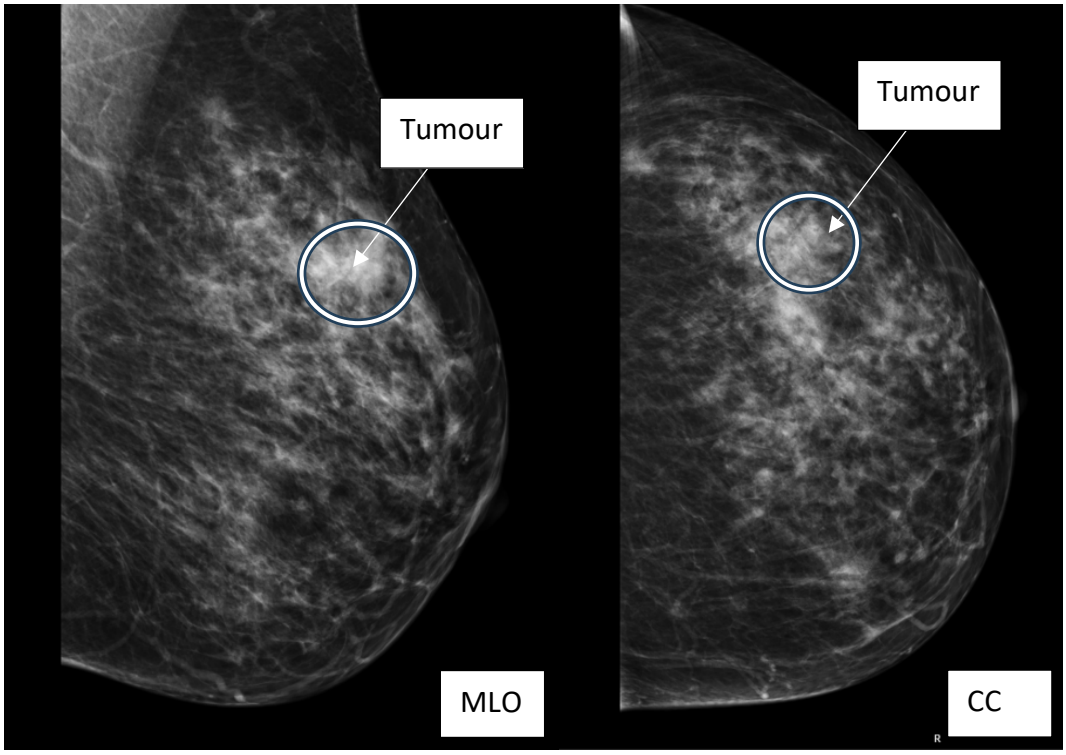


Figure 27 TNBC002 mammography. *TNBC002 left breast, standard two view representations (Left MLO view, Right CC view). Areas highlighted represent detected areas of suspicion.*

TNBC002 was then discussed at the breast MDT and both biopsied areas were found to represent a TNBC with the 10 o'clock lesion being a Grade 2 invasive ductal adenocarcinoma and the 7 o'clock lesion being a Grade 3 invasive ductal adenocarcinoma. TNBC002 went onto have an MRI to exclude other disease sites, this revealed the index tumour in the upper out quadrant at the 10 o'clock position (TNBC002A) and a further area at the 7 o'clock position (TNBC002B) (**Figure 28**). Prior to the patient commencing NACT she was approached to take part in the research

study and had been found to be negative for BRCA1/2. The timeline of research participation is detailed in Table 3. Visit 1 was undertaken prior to the patient starting NACT. TNBC002 is currently undergoing NACT with carboplatin, paclitaxel and pembrolizumab which will be followed by epirubicin, cyclophosphamide and pembrolizumab and has yet to complete the research study at the time of submission.

Table 3 Timeline of TNBC002

Visit Number	Visit Date	Intervention
1	3/4/23	Imaging and core biopsy
2	12/6/23	Imaging and core biopsy
3	Pending	Imaging alone
4	Pending	Surgery

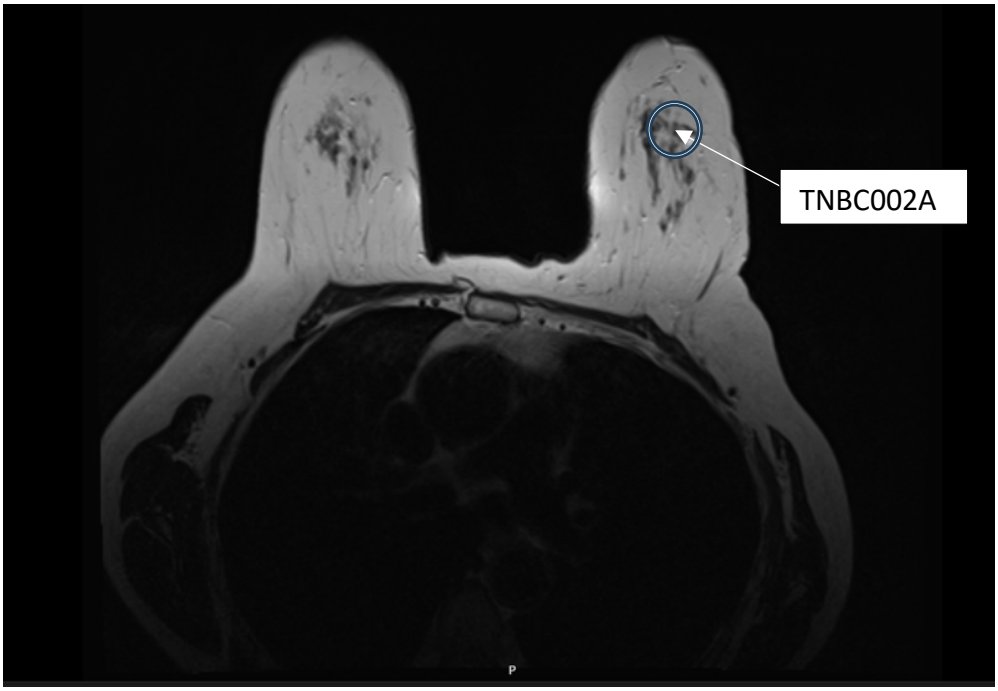


Figure 28 Contrast MRI both breasts. *Left breast demonstrating TNBC002A area (TNBC002B not visualised in this plane)*

3.5.1 Immunohistochemistry

TNBC002 has attended visits 1 and 2 at which time tissue samples have been taken. Each has been processed by MTW pathology department prior to IHC being performed (**Figures 29, 30, 31**) to test for markers of proliferation (Ki67, EGFR), angiogenesis (VEGFR, VEGFA, CD31, CD34) and vascular mimicry (absence of CD31, PAS staining and H&E).

3.5.1.1 TNBC002A

The IHC performed on TNBC002A during the visits demonstrated clear differences between the amount of staining seen on progressive visits in many of the antibodies tested. As yet this patient is yet to complete the research study and the effects of further NACT and immunotherapy are still pending. Currently between pre-chemotherapy/immunotherapy (visit 1) and midway through chemotherapy (visit 2) we have observed:

Ki67 – At visit 1 there were high detectable rates of Ki78 with a moderate degree of background staining. At visit 2 this staining had decreased but there was still evidence of Ki67 present.

EGFR – Present at visit 1 in multiple areas which appears to have largely disappeared at visit 2.

VEGFR – Clear staining visible at visit 1 which decreased by visit 2 with no clear staining around the ducts noted.

VEGFA – High degree of positive staining at visit 1 and minimal staining noted at visit 2.

CD34 – High degree of staining throughout visit 1 and some minimal reduction in staining evident by visit 2.

CD31 – Staining noted throughout both visits with no discernible difference.

In terms of the presence of basal antibodies in TNBC002A there were no detectable signs of this at visit 1 and no further visits were tested. Thus, this area likely phenotypically does not represent an area of basal like disease.

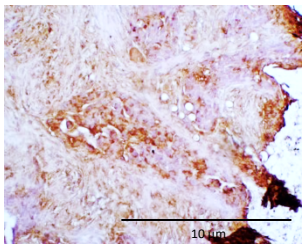
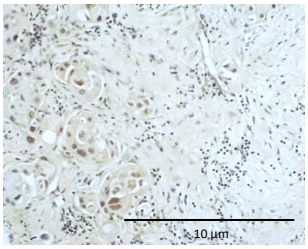
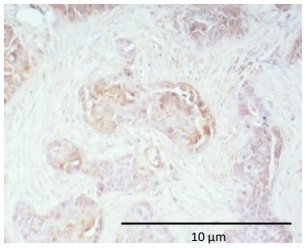
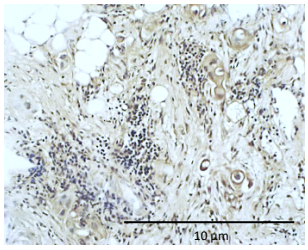
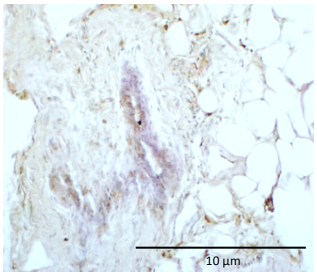
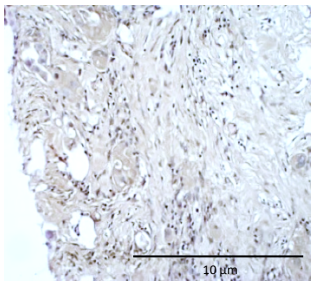
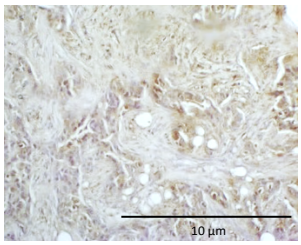
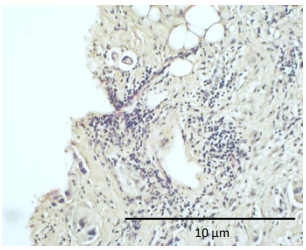
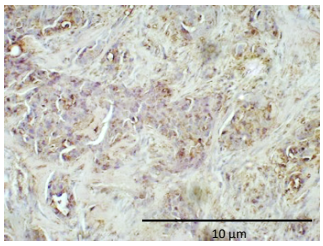
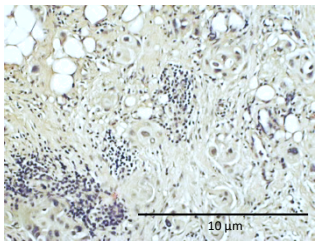
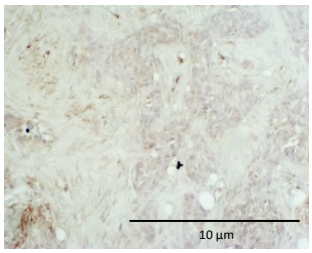
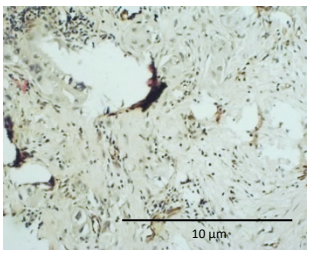
Antibody	Visit 1	Visit 2	Visit 4
Ki67 1:100 100x			Not yet attended
EGFR 1:100 100x			Not yet attended
VEGFR 1:100 100x			Not yet attended
VEGFA 1:100 100x			Not yet attended
CD34 1:100 100x			Not yet attended
CD31 1:100 100x			Not yet attended

Figure 29 – TNBC002A– Immunohistochemistry staining of markers of proliferation (EGFR, Ki67), markers of angiogenesis (CD34, CD31, VEGF, VEGF-R) and vascular mimicry. *Dilutions of each marker and magnification in figure. All visualised as per materials and methods.*

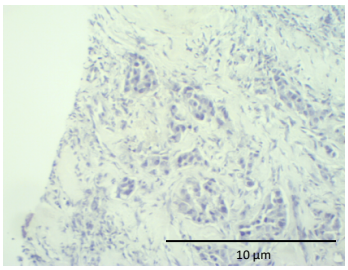
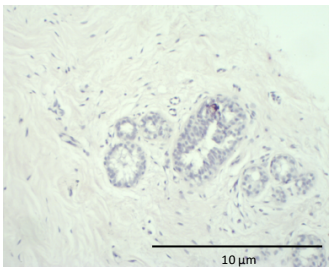
Antibody, dilution and magnification	Visit 1	Visit 2	Visit 4
TNBC002A No Primary antibody 100x			Not yet attended

Figure 30 **–TNBC002A – Immunohistochemistry with no primary antibody (negative control) with H&E counterstaining.** No evidence of improper staining. All visualised *as per materials and methods.*.

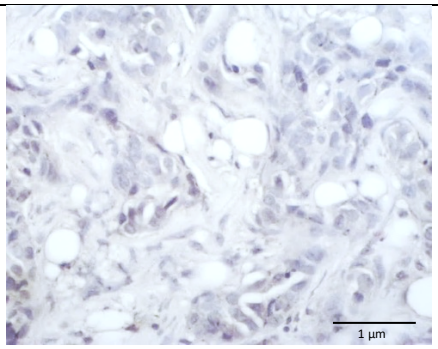
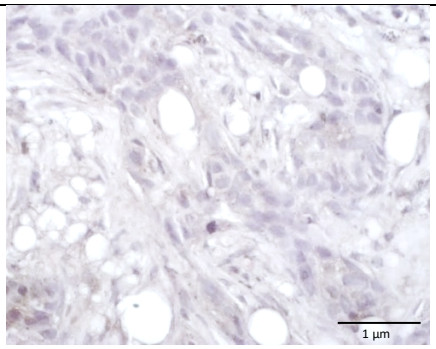
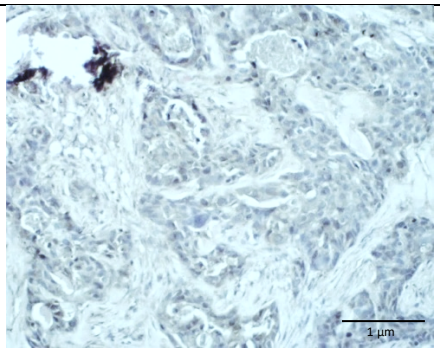
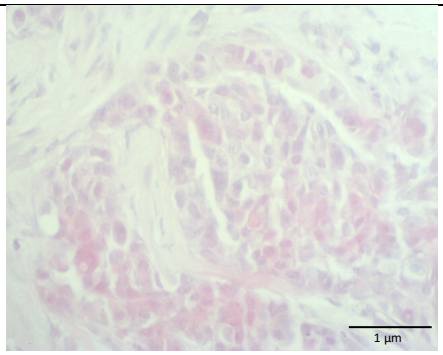
Antibody	Visit 1
CK5 1:75 200x	
Ck6 1:500 200x	
Ck14 1:100 200x	
No primary antibody (negative control) 200x	

Figure 31 TNBC002A **– basal antibodies (CK5, CK6, CK14)** IHC tested at Visit 1 to assess for phenotype in this sampled area and negative control with no primary antibody. All visualised *as per materials and methods.*

PAS staining was also undertaken from each visit attended. There is no evidence of PAS positive staining, endothelial cells are present and as such there is no evidence of vascular mimicry **(Figure 31).**

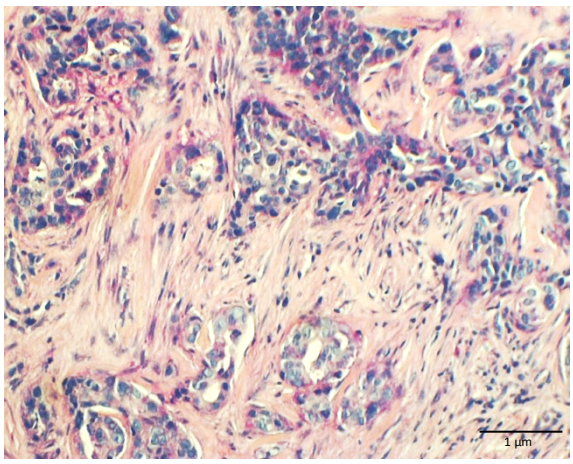
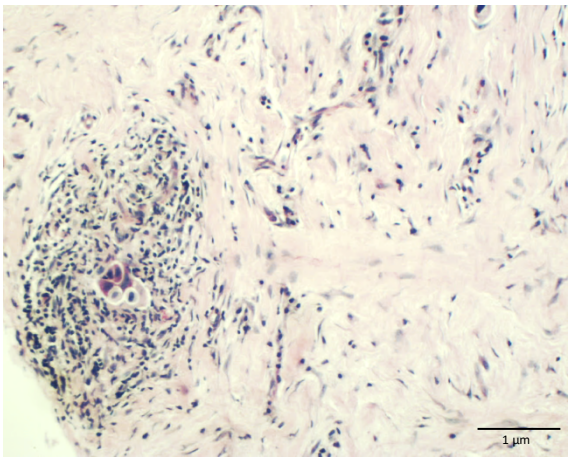
TNBC002 A	PAS staining 1:100
Visit 1 200x	
Visit 2 200x	
Visit 3 200x	Not yet attended

Figure 32 -TNBC002A PAS staining undertaken at each visit to assess for the presence of vascular mimicry (VM). No VM demonstrated in this sampled area. All visualised *as per materials and methods.*

3.5.1.2 TNBC002B

The IHC performed on TNBC002B during the two visits demonstrates some differences however the biopsy taken on the second visit represents only a small focus of breast tissue with a single duct so interpretation is difficult at this stage (**Figure 33, 34, 35**).

Ki67 – Present in multiple areas at visit 1. Some background staining around the visible duct at visit 2 but unable to detect other areas in this sample.

EGFR – Not present at visit 1 and only background staining seen at visit 2. No evidence of EGFR nuclei staining.

VEGFR – Not present at visit 1 and only background staining seen at visit 2 around the only duct present.

VEGFA – Strong positive staining seen throughout this sample during visit 1. Ongoing staining seen at visit 2.

CD34 – Positive staining seen at visit 1 and within the limitations of the sample at visit 2 staining still evident.

CD31 – small areas of staining clearly visible at visit 1. Only small area of staining around the duct in visit 2 and likely artefact.

There was no evidence of basal type present at TNBC002B as evidenced by lack of CK5, CK6, CK14 (**Figure 35**).

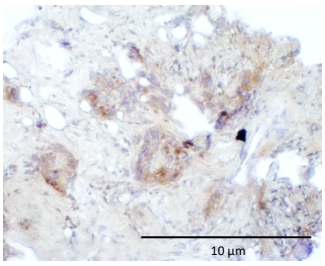
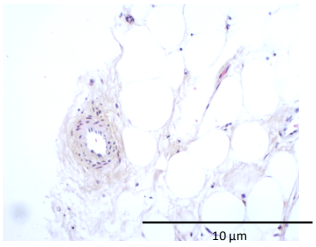
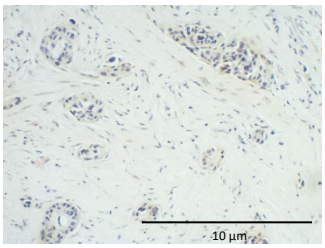
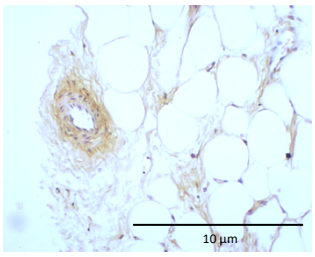
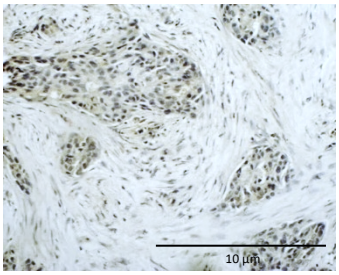
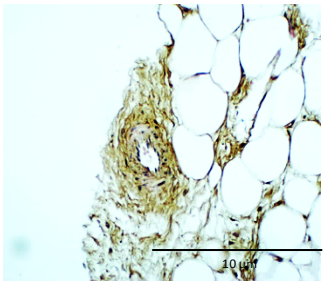
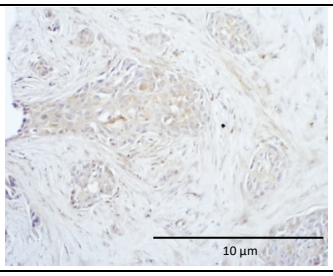
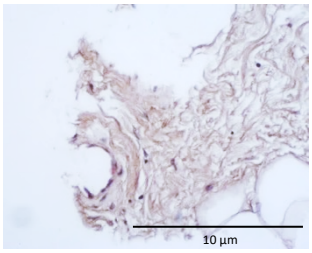
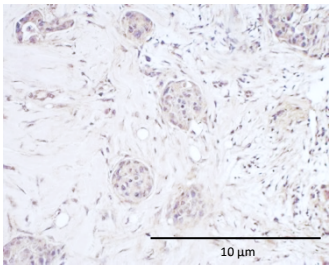
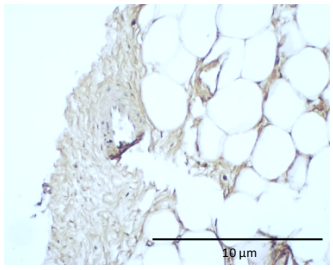
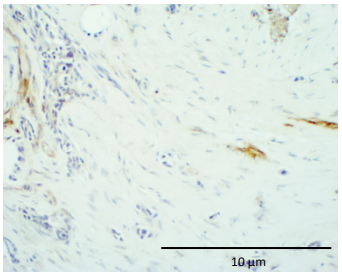
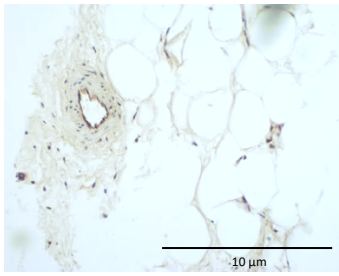
Antibody	Visit 1	Visit 2	Visit 4
Ki67 1:100 100x			Not yet attended
EGFR 1:100 100x			Not yet attended
VEGFR 1:100 100x			Not yet attended
VEGFA 1:100 100x			Not yet attended
CD34 1:100 100x			Not yet attended
CD31 1:100 100x			Not yet attended

Figure 33 – ***TNBC002B– Immunohistochemistry staining of markers of proliferation (EGFR, Ki67), markers of angiogenesis (CD34, CD31, VEGF, VEGF-R) and vascular mimicry.*** *Dilutions of each marker and magnification in figure. All visualised as per materials and methods.*

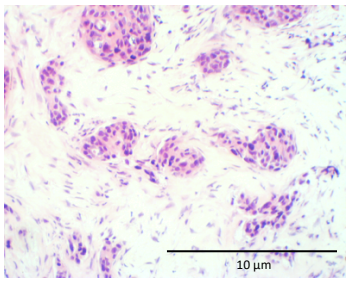
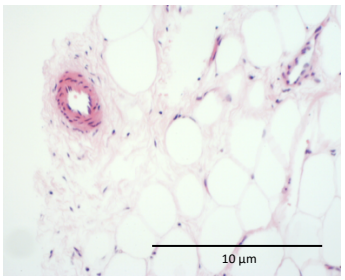
Antibody, dilution and magnification	Visit 1	Visit 2	Visit 4
TNBC002B No Primary antibody 100x			Not yet attended

Figure 34 – TNBC002B Immunohistochemistry with no primary antibody (negative control) with H&E counterstaining. *No evidence of improper staining. All visualised as per materials and methods.*

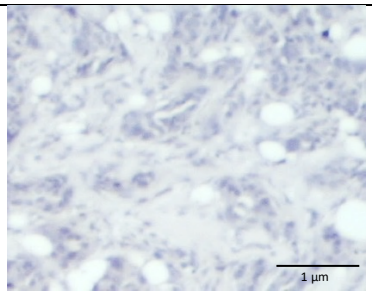
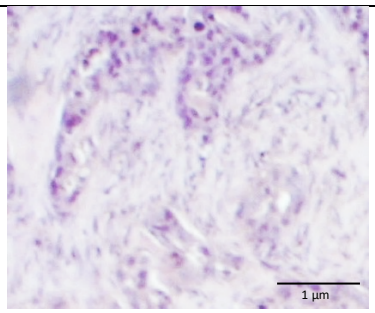
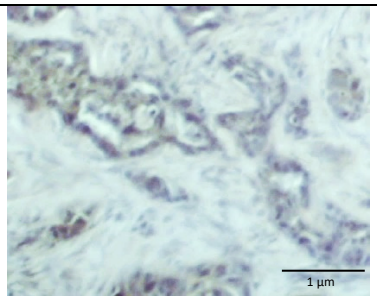
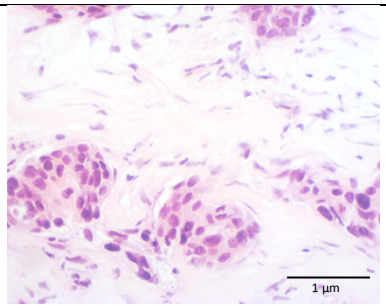
Antibody	Visit 1
CK5 1:75 200x	
Ck6 1:500 200x	
Ck14 1:100 200x	
No primary antibody (negative control) 200x	

Figure 35 TNBC002B – basal antibodies (CK5, CK6, CK14). *IHC tested at Visit 1 to assess for phenotype in this sampled area and negative control with no primary antibody. All visualised as per materials and methods.*

PAS staining performed on visit 1 and 2 has not demonstrated any loss of endothelial cells and therefore no evidence of vascular mimicry (**Figure 36**).

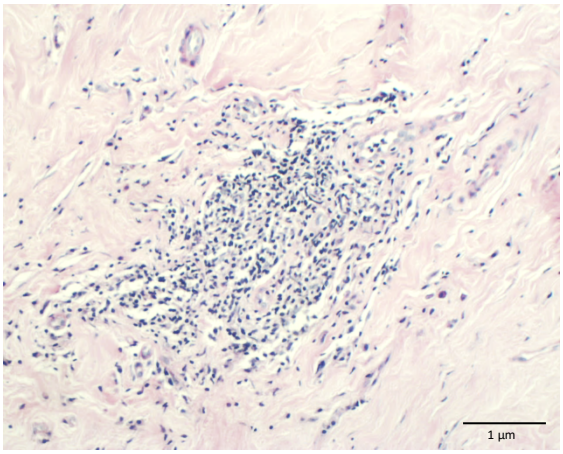
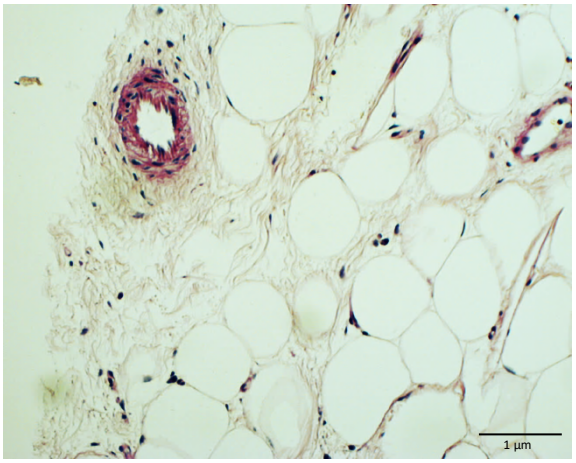
TNBC002 A	PAS staining 1:100
Visit 1 200x	
Visit 2 200x	
Visit 3	Not yet attended

Figure 36 TNBC002B PAS staining undertaken at each visit to assess for the presence of vascular mimicry (VM). *No VM demonstrated in this sampled area. All visualised as per materials and methods.*

3.5.2 Imaging

High-resolution CEUS was undertaken of the areas of disease at the time intervals discussed. Again, the two distinct areas of disease were identified and correlated to areas biopsied and labelled as TNBC002A (**Figure 37**) and TNBC002B (**Figure 38**). This patient is yet to complete the research study at the time of writing.

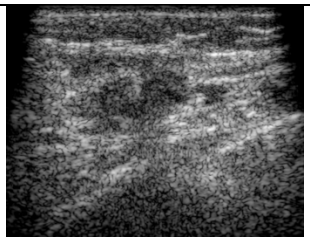
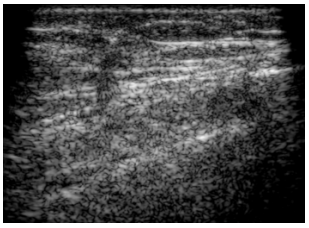
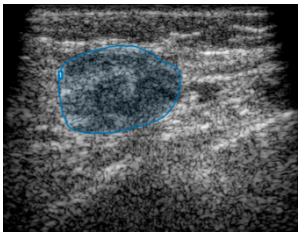
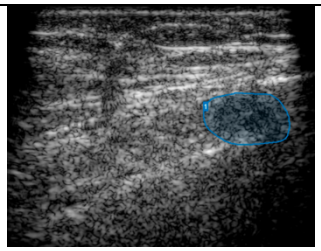
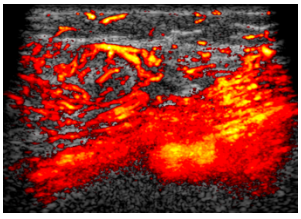
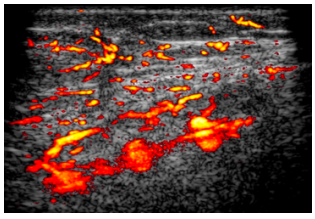
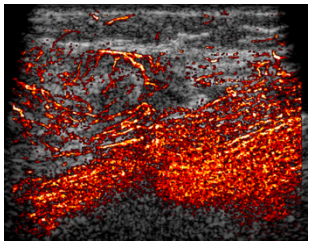
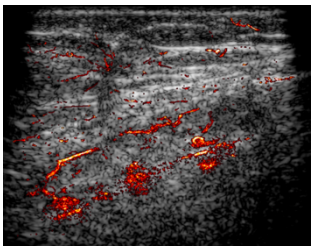
Imaging modality	Visit 1	Visit 2	Visit 3
Standard B mode ultrasound			Not yet attended
Standard B mode ultrasound with ROI			Not yet attended
Contrast enhanced ultrasound			Not yet attended
Super localisation of microbubbles			Not yet attended

Figure 37 – Imaging TNBC002A - Standard B mode ultrasound with and without highlighted region of interest. CEUS area of whole field of view with further super localisation of microbubbles around region of interest which appears to reduce during subsequent visits.

TNBC002A radiologically demonstrated a large area of disease on NHS standard imaging. This was identified using the Vantage 256^{TM} Research platform as demonstrated in Figure 37. Utilisation of the high-resolution CEUS after injection of microbubbles demonstrated a large area of vascularity around the tumour. Whilst the majority of the area highlighted was distant from the ROI a significant amount was directly around the tumour. This area demonstrably reduced on the second visit as evidenced by high-resolution CEUS and the super localisation. Therefore, from these two visits we can discern that there has been a reduction in size of the ROI and also a reduction in the number of vascular channels directly supplying the tumour.

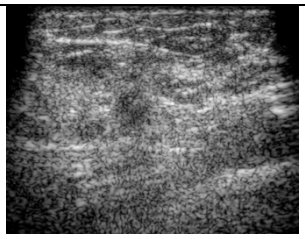
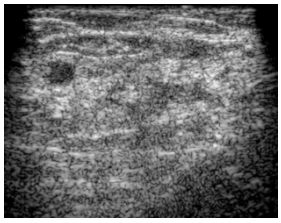
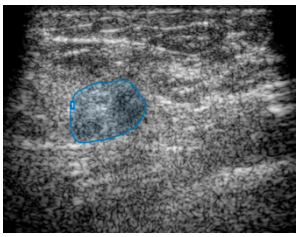
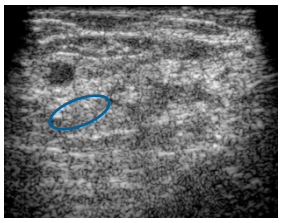
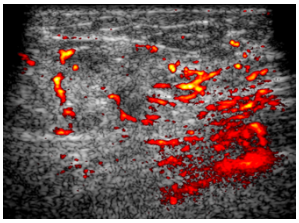
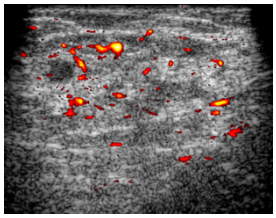
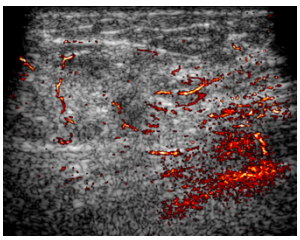
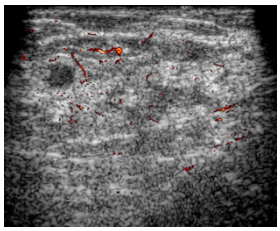
Imaging modality	Visit 1	Visit 2	Visit 3
Standard B mode ultrasound			Not yet attended
Standard B mode ultrasound with ROI			Not yet attended
Contrast enhanced ultrasound			Not yet attended
Super localisation of microbubbles			Not yet attended

Figure 38 – **Imaging of TNBC002B -** Standard B mode ultrasound with and without highlighted region of interest. CEUS area of whole field of view with further super localisation of microbubbles around region of interest which appears to reduce during subsequent visits.

TNBC002B demonstrated a smaller size of the tumour with a representative smaller area of vascularity around the ROI. Nevertheless, this area had decreased in size by visit 2 such that on super localisation there were hardly any microbubbles appreciable at the ROI.

TNBC002 is still part of the research study and is on course to attend her final chemotherapy visit in late October 2023 (there have been a number of delays to her neoadjuvant treatment). TNBC002 was commenced on immunotherapy and has since completed this regimen. The final dates for the research visit and surgical intervention are yet to be confirmed.

Chapter 4: Discussion

4.1 Introduction

This clinical feasibility research study sought to assess whether the combined use of high-resolution CEUS and immunohistochemical markers of angiogenesis (CD34, CD31, VEGF, VEGF-R), proliferation (EGFR, Ki67) and vasculogenic mimicry (H&E, PAS staining, absence of CD31) could provide a more accurate way to monitor TNBC tumours versus standard CEUS in patients undergoing neoadjuvant chemotherapy (NACT) and could provide improved specificity for detecting pCR. Initial IHC was performed on the HCC38 cell line prior to progressing to patient samples to assess for a response to NACT as well as defining the phenotype of each tumour area (through the presence of CK5, CK6, CK14). The two recruited patients (TNBC001, TNBC002) were each found to have two separate areas of disease (TNBC001A, TNBC001B, TNBC002A, TNBC002B) with each analysed separately. TNBC001 has completed the research study whilst TNBC002 is yet to attend for her final imaging appointment and surgical intervention.

4.2 Immunohistochemistry

4.2.1 Phenotype

Initial phenotype IHC testing was undertaken to assess for the presence of specific markers that may indicate the basal phenotype (CK5, CK6, CK14). There are currently no set markers that are used to define a basal-like phenotype of TNBC however CK5, CK6 and CK14 have been shown to be the most reliable markers as a diagnostic tool. Other markers have been identified in the literature to identify basal-like breast cancers and include SMA, P-cadherin, p63 or c-kit antigen(38). TNBC001A stained positive for all of these whilst TNBC001B did not stain for CK14, this was confirmed

with negative controls on patient samples. TNBC002 showed no evidence of positive staining in either area which again was confirmed with negative controls.

Establishing the phenotype of the TNBC tumours is clinically relevant as these tumours are treated with NACT and surgery and no adjuvant treatment, therefore determining the specific phenotype can lead to improved management and surveillance. The basal subtype of TNBC in the majority of cases is characterised by high mitotic indices, the presence of central necrotic zones and a high histological grade (37). Patients newly diagnosed with TNBC would likely welcome more information regarding their diagnosis and defining a specific phenotype may influence their decision-making when being offered specific treatments that in the future may be individually tailored to their specific TNBC subtype.

4.2.2 Cell proliferation markers

The role of Ki67 as a marker for cell proliferation has been well established in previous studies (18) and its presence in normal healthy tissues has been reported at very low levels. However, the international Ki67 Breast Cancer Working Group has accepted that Ki67 as a prognostic marker has limited clinical validity on its own (39). As such its role to monitor response to NACT in TNBC in combination with other immunohistochemical markers has not been established in the literature.

In this study, we were unable to demonstrate any significant evidence of positive staining for Ki67 in TNBC001A whilst some evidence was seen in TNBC001B in multiple nuclei which decreased over subsequent visits until there were no detectable levels at the time of surgical excision. In addition, as noted from imaging undertaken; both disease areas had central necrosis indicative of a rapidly growing tumour. It is likely that TNBC001A had a similar IHC profile to that of TNBC001B but due to the level of

necrosis detection was hampered. Conversely, Ki67 was found to be strongly positive in TNBC002 in both areas analysed which had reduced once NACT had commenced as evidenced by the results seen in visit 2. TNBC002 analysis of Ki67 at visit 4 will be an important result to evaluate whether it will play a role in monitoring NACT in TNBC patients.

EGFR had a similar appearance on IHC for both TNBC001 and TNBC002 where it was detectable throughout NACT. In TNBC001 there were minimal levels of EGFR staining present on the final surgical specimen. This would tally with the results from the final histopathological diagnosis that there was no detectable cancer present. EGFR is known to have a role in cell proliferation but the mechanisms involved are varied and isolated targeted therapy thus far has not been shown to be beneficial to patients with TNBC(40).

4.2.3 Angiogenesis markers

The angiogenesis markers analysed included VEGFR, VEGFA, CD34 and CD31. In TNBC001A and TNBC001B, there was evidence of staining for these markers on IHC at visit 1 and 2 with only CD34 remaining persistent at the time of surgical resection. Whilst in TNBC002; VEGFR, VEGFA, CD34, and CD31 was clearly evident at visit 1 and 2 with the results of visit 4 and the final surgical specimen providing more information regarding the monitoring of these markers when available.

VEGFA and its receptor VEGFR are known to have an important role in angiogenesis and this interaction represents a major pathway to activate angiogenesis(41). VEGFA and VEGFR were clearly present with a high amount of staining at the initial visits and its reduction during NACT may be an indication of a slowing/ stopping of angiogenesis occurring in these patients. Previous studies have highlighted the VEGF/ VEGFR system

to be an important target for antiangiogenic treatments and thus monitoring this during NACT may provide a simple effective way to assess response (42)

CD34 is known to be a specific marker of vascular endothelial cells and is sensitive to tumour angiogenesis (43). High levels of CD34 indicate a high microvessel density which can lead to an increased chance of local invasion and metastasis. CD34 remained raised throughout NACT and was detectable at the time of surgical intervention in both TNBC001A and TNBC001B, whilst it was persistent in TNBC002 thus far. Angiogenesis is a multifactorial process and not solely reliant on CD34 as such raised levels post NACT are not an indication of a higher risk of recurrence.

CD31 was found to be initially present in both TNBC001 and TNBC002 before being undetectable post-NACT in TNBC001. This again mirrors the findings of pCR and indicates a decrease in angiogenesis and the findings from TNBC002 will be important to verify this result.

4.2.4 Vascular mimicry

As previously described vascular mimicry (VM) is an alternative method of tumour microcirculation independent of angiogenesis. These vascular channels do not display markers of angiogenesis such as CD31 and on PAS staining are found to be positive. In TNBC001A and TNBC001B there was evidence of CD31 staining and PAS staining was found to be negative and as such there was no evidence of VM in this patient. This may be unexpected as previous studies have demonstrated that a promoter of VM is that of hypoxia; TNBC001A had radiological evidence of central necrosis which would lead to hypoxia of the tissue and increase the risk of VM(44). It is likely that as this necrosis developed rapidly VM was not established at any point. TNBC002 has thus far shown no evidence of VM. This fits with the pattern seen in terms of IHC as there is evidence

that markers of angiogenesis are present and this would likely preclude the need for VM as a process required for cancer progression. The final IHC findings for VM in TNBC002 will give more information as unfortunately there have been multiple delays in NACT and subsequently an increased wait until surgical intervention.

4.3 Imaging

In both patients, NHS standard imaging was performed in the form of mammograms and B-mode ultrasonography (**Figures 21, 25**) and pre-NACT treatment MRI (**Figures 22,26**). TNBC001 was found to have central necrosis in both areas, whilst TNBC002 had an overall larger area of disease. High-resolution CEUS was then performed and was able to identify the pathway of the injected microbubbles in the vasculature surrounding the tumour and ROI. Following this, post-processing imaging analysis was undertaken and super localisation of these microbubbles was visualised in both TNBC001 and TNBC002 and the microvasculature was clearly demonstrated (**Figures 25,26, 37,38).**

Angiogenesis is a key component in the development of new blood vessels in solid tumours as they outgrow their native blood supply. On visualisation of the microbubbles, it is evident that the pathway undertaken around the tumours in particular represents non-hierarchical branching, erratic vessel shape and leaky vessels which have all the features of pathological angiogenesis. These microbubbles are smaller than capillaries and as such when injected intravenously can travel through these pathological vessels and highlight vasculature around these tumours. Therefore, we were able to see evidence of these new vessels using the high-resolution CEUS.

TNBC001A vasculature is highlighted in Figure 25 and shows at visit 1 that around the ROI there are a number of feeding vessels which are persistent on super localisation

imaging. These vessels began to decrease during NACT and by visit 3 on completion of NACT the feeding vessel around the ROI had completely disappeared. The disappearance of these vessels and the final findings of pCR have shown that the NACT was effective at treating the tumour in this region. Similarly, TNBC001B demonstrated an even more haphazard pattern of microbubble distribution around the ROI (**Figure 26**) which is likely the result of this region having less central necrosis than TNBC001A. Through NACT these aberrant vessels around the ROI had begun to decrease and by visit 3 all of these had disappeared. The remaining normal vasculature adjacent to the tumour remained during each visit and demonstrated that the NACT did not affect these vessels as they were not pathological in nature.

In TNBC001 when the imaging findings of the super localisation of microbubbles are paired with the IHC findings it is clear that upon completion of NACT, this patient had had a complete response which is why at surgical intervention no residual cancer was noted in the mastectomy specimen. In contrast, TNBC002 highlighted in high-resolution CEUS that there was a large influx of microbubbles surrounding the tumour ROI especially evident in TNBC002A (**Figure 37**). Subsequent imaging at visit 2 demonstrated a reduction in these vascular channels and a consequent shrinking of the tumour and ROI. Further imaging is required at visit 3 to determine if this decrease in the size of the tumour and microbubble channels continues. The imaging undertaken thus far has demonstrated a reduction in the apparent aberrant vasculature around the ROI in both TNBC002A and TNBC002B. Indeed the smaller area at TNBC002B has almost had a complete reduction in the vasculature around the ROI whilst TNBC002A still has a large proportion of these vessels present which is likely a reflection of the size of the initial tumour.

As previously mentioned, this patient was additionally commenced on pembrolizumab in addition to standard NACT and it will be interesting to see if she also achieves pCR. As pembrolizumab is an immune checkpoint inhibitor it has been shown in studies (KEYNOTE-522) to give an increased rate of pCR than NACT alone in patients with TNBC who express PD-L1.

Overall, high-resolution CEUS has been able to highlight the pathway of the intravenously injected microbubbles and also been able to reliably re-evaluate them at subsequent visits and demonstrate that they have decreased during both patients' NACT journey. Further information will be required to corroborate these findings in the form of future research imaging; NHS standard imaging in the form of final treatment MRI as well as the results from histological examination of the final surgical specimen in TNBC002.

4.4 Limitations

This research study represents only a small sample of patients affected by TNBC. In an ideal situation, more patients would have been included during the recruitment phase of this study however as TNBC represents only a small proportion of new diagnoses of breast cancer as well as strict inclusion and exclusion criteria recruitment has been challenging. The target number for recruitment for this research study was N=5 however despite a proactive research team many patients approached whilst initially interested in the draw of research unfortunately declined for a number of reasons. The most frequent explanation given for non-participation was the requirement for further biopsies which may be a traumatic/ painful situation for many.

In addition to difficulties with recruitment, another problematic issue with this study is the time taken for NACT to be completed. In the UK NACT does not consist of a set

regime for all patients and as such many experience complications or issues with ongoing NACT resulting in delays to treatment. TNBC001 experienced minor delays to her NACT which resulted in a greater time interval from recruitment to the study to surgical intervention than expected. Similar findings have been noted in TNBC002 where unfortunately she has experienced minor/moderate complications during NACT resulting in delays to further cycles and an expected end to NACT in October 2023.

Finally, another confounding factor during the study was the addition of Pembrolizumab to the treatment regimen of patients newly diagnosed with TNBC. TNBC001 began NACT prior to this being approved by NICE whilst TNBC002 benefited from this addition to her treatment pathway. Studies have shown the beneficial nature of PD-L1 inhibitors with an increased rate of pCR. Thankfully, TNBC001 was found to have pCR and it is hoped that TNBC002 will also have the same findings (with encouraging imaging shown in the research study). This addition of pembrolizumab does mean that comparing the information gained between these two patients will be difficult and does mean that further studies will need to be undertaken on a larger scale to highlight the efficacy of high-resolution CEUS.

4.5 Future work

This study has laid the basis for future large-scale studies to investigate the role of high-resolution CEUS and immunohistochemical markers in monitoring TNBC disease response during NACT. Future studies would likely benefit from being undertaken concurrently at multiple centres to eliminate/ mitigate the difficulties found in this study. Additionally, if high-resolution CEUS were validated for use during NACT without the need for repeat biopsies then there may be more interest from potential patients to be part of a research study.

Chapter 5: Conclusion

The results from this research study have demonstrated that the use of high-resolution CEUS in conjunction with markers of cell proliferation, angiogenesis and vascular mimicry is a safe and effective tool to monitor NACT in TNBC.

TNBC001 and TNBC002 both have raised no major issues with the research protocol and have been advocates of the imaging to other patients. The imaging findings in both patients have shown a clear change in the tumour microvasculature during NACT (whilst final results of TNBC002 are pending) with these findings being reflected in the change in the selected IHC markers. Thus far the results have shown that the tumours in both patients have been driven by angiogenesis and in this small cohort there has been no evidence of VM. Finally, there has been no evidence that the response to NACT has been influenced by the phenotype of the tumour or mutations in *BRCA1/2.*

Overall, it is clear that the findings from this study are encouraging and warrant further investigation on a larger scale to provide greater evidence for the need for high-resolution CEUS during NACT in TNBC patients.

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Chapter 6: Appendices

6.1 Consent form

Consent Form

IRAS ID: 301345

Centre Number:

Study Number:

Participant Identification Number for this trial:

CONSENT FORM

Title of Project: Imaging of tumour microvasculature using high-resolution contrast enhanced ultrasound (CEUS) together with markers of proliferation/ angiogenesis/ vascular mimicry to characterise response to neoadjuvant chemotherapy in triple negative breast cancer.

Name of Researcher:

Please initial box

1. I confirm that I have read the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand where it is relevant to my taking part in the research, that sections of my medical notes may be looked at by regulatory authorities or by the NHS Trust. I understand that anonymous data collected during the study will be looked at by individuals from Imperial College London, The University of Kent, regulatory authorities, or the NHS Trust. I give permission for these individuals to have access to my anonymous data. ☐
4. I agree for my GP to be informed of my participation in the study. ☐
5. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers including those outside of the U.K. ☐

6. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person seeking consent	Date	Signature

6.2 Patient information leaflet

Patient Information Sheet

Imaging of tumour microvasculature using high resolution contrast enhanced ultrasound (CEUS) together with markers of proliferation/ angiogenesis/ vascular mimicry to characterise response to neoadjuvant chemotherapy in triple negative breast cancer.

We invite you to take part in a research study

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve.

Please take time to read the following information carefully and discuss it with others such as your friends and family if you wish.

Ask us if there is anything that is not clear or if you would like more information.

Take time to decide whether or not you wish to take part.

Thank you for reading this.

How to contact us:

If you have any questions about this study, please contact **Mr Jaideep Rait** during working hours on the following contact number or email address:

Tel: 01622 939736 (x39736)

Email: j.rait@nhs.net

Contents

15. Who has reviewed the study?

1. What is the purpose of this study?

Around 50,000 people per year are told that they have breast cancer in the UK. Triple negative breast cancer (TNBC) is a specific type of breast cancer that is not hormone (oestrogen and progesterone) dependant and accounts for up to 15% of these cases. Treatment for TNBC almost always includes chemotherapy, often given before surgery to try and shrink the tumour.

It can be difficult to track how the cancer is responding to chemotherapy using routine tests such as standard ultrasound or breast MRI. New high resolution ultrasound technology using tiny injected bubbles (microbubbles) holds the promise of improved monitoring by allowing the blood vessels inside the tumour to be seen. Combining this with evaluation of microscopic changes in the tumour will give an insight into how tumour blood flow changes during chemotherapy. Early changes in the blood flow after chemotherapy starts may be a good indicator of whether the cancer will shrink or not. It may even be able to predict those cancers that will shrink away completely or or even whether the cancer will keep growing despite the treatment.

The research is part of Mr Rait's Masters degree at The University of Kent. Mr Rait is a trainee Breast Surgeon (registrar) supervised by the Consultant Breast Surgeon Miss Karina Cox, Consultant Oncologist Dr Catherine Harper-Wynne and Consultant Pathologist Dr Sonia Saw as well as Professor Michelle Garrett at the University of Kent and Professor Mengxing Tang at Imperial College London.

If successful, this research will lay the foundation for a larger study to see if the new technology can replace the existing standard imaging tests used to diagnose and monitor TNBC during chemotherapy.

2. Why am I being chosen?

We have chosen to approach you to take part in the study because you have recently been diagnosed with triple negative breast cancer (TNBC). As part of your treatment, your specialists have recommended that you have chemotherapy first followed by surgery.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. It is also important that you are not taking part in any other research studies while you are involved with this study. Your involvement with this study ends after you have surgery.

4. What will happen to me if I take part?

Your breast cancer treatment will happen normally. The only difference will be that you will be monitored more closely during chemotherapy with the high-resolution contrast test and 2 extra biopsies will be taken from the cancer. We need to take the

extra biopsies to see whether the ultrasound blood flow changes accurately reflect what is happening at the microscopic level inside the tumour. Your GP will be informed of your involvement in the study unless you instruct us not to do so.

If you agree to take part, you will need to come back for **three** extra visits to your breast unit's ultrasound department to have the new high-resolution contrast ultrasound test and extra biopsy samples taken will be taken on **two** of those visits. These visits will take place: **(1)** before starting chemotherapy (ultrasound and biopsy), **(2)** between your 2nd and 3rd dose of chemotherapy (ultrasound and biopsy) so we can assess what happens in the first few weeks after starting chemotherapy and **(3)** 2 weeks after chemotherapy finishes (ultrasound only).

A member of the research team will insert a cannula (small tube) into a vein in your arm before the test and inject the ultrasound contrast.

A consultant breast radiologist will perform the new ultrasound test with a member of the research team and a biomedical engineer from Imperial College also present for the procedure.

The research test should take about 45 minutes.

After the images are obtained, on visits **(1)** and **(2)** local anaesthetic will be injected, and a biopsy of the tumor taken. On visit **(1)**, some of the tissue will be transported to UCL genomics in London to see if the tumour is carrying genetic mutations in the breast cancer linked genes BRCA1, BRCA2, CHEK2 and PALB2. The rest of the tissue and all the biopsy sample on visit **(2)** will be sent firstly to the hospital's pathology department for processing and then transported to the School of Biosciences at the University of Kent for the research tests to be performed as soon as they arrive. After the research tests have been completed, the biopsy material will be disposed of in accordance with the UK Human Tissue Act.

After the test, you will be asked to complete a satisfaction questionnaire by a member of the research team. You can complete this before going home or take it home and post it back to the research team.

Following these three visits and after finishing chemotherapy you will have your breast surgery as normal standard of care.

5. What do I have to do?

Before and after the new ultrasound test, you can behave normally and take regular medications. It is fine to drive yourself or use public transport to get to the appointment, but you may prefer to have someone with you. We can offer you reasonable travel expenses or a waiver for your parking costs.

6. What is the drug and intervention that is being tested?

The drug is called SonoVue and it is a microbubble ultrasound contrast agent. Microbubbles are tiny gas filled bubbles that are smaller than a red blood cell. The bubbles reflect the ultrasound beam to show structures in the body. The hexafluoride gas in the microbubbles is quickly and harmlessly removed from the body by the lungs. They have been used for many years to look at blood flow in arteries and internal organs by injecting them into the bloodstream.

This research uses SonoVue microbubbles injected into the bloodstream (up to 5 ml). People with serious heart, lung or kidney disease should not have microbubbles injected into the body. Likewise, people who have recently had a thromboembolism

(major blood-clot) or whose blood is prone to clotting (hyper-coagulation disorder) should not have injected microbubbles.

The equipment being used to develop the new ultrasound test is made up of a research ultrasound machine, which is safe to use in studies on people. This machine will be attached to a normal ultrasound probe (already in use on patients) and this lightweight probe is the only part of the equipment that will be in contact with your skin. An experienced radiology doctor will perform the test ultrasound scan.

The extra biopsies for the research are taken in the same way as the biopsy you had when your breast cancer was first diagnosed.

7. What are the side effects of the biopsy and having the new ultrasound test?

Injecting SonoVue microbubbles into the bloodstream is safe but there are some uncommon (1/1000 to 1/100) side-effects that could occur immediately after the injection such as headache, dizziness, tingling of the skin, a funny taste in the mouth, skin flushing, sickness, abdominal pain, skin rash, discomfort in your chest, feeling hot and skin redness at the site of the injection. Rare (1/10,000 to 1/1000) side effects include allergic reaction, blurred vision, low blood pressure, itching of the skin, back pain, chest pain and fatigue.

There are no known long-term side effects of SonoVue microbubbles.

Possible side effects of the biopsy include blood collection (haematoma), bruising and soreness.

8. What are the possible disadvantages and risks of taking part?

You will need to come back to the breast unit for three extra visits, which may be inconvenient.

9. What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get might help improve the treatment of people with triple negative breast cancer in the future.

You will be monitored more closely than normal as you go through the chemotherapy. The new ultrasound test uses cutting edge technology called ‘high-resolution contrast enhanced ultrasound’ that is much more powerful than a normal ultrasound. It is therefore possible that during the research test, when the radiology doctor scans your breast they may find the breast tumour is bigger than initially measured or there is more than one cancerous area in the breast. If this happens, this information will be given immediately to the doctors involved in your care as it may affect your treatment plan. They will then organise another appointment with you to talk about the findings. Additionally, the information gained from the biopsy samples as well as the new ultrasound test may show that there has been a greater or lesser response to the chemotherapy than expected and this information will be relayed immediately to the doctors involved in your care.

We are also planning to see if the cancer has mutations in the BRCA1/2 genes and this information will be given to your oncology doctor as it may change the type of

chemotherapy that they give you. If the cancer comes back in the future, either as a recurrence in the breast or armpit or as secondary breast cancer, knowing whether the original cancer was carrying mutations in the BRCA1/2 genes, could affect the type of treatment offered to you by your doctors.

Sometimes during the course of a research project, new information becomes available about the intervention/ drug that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your research doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

10. What happens when the research study stops?

After the extra visits for biopsy samples and high-resolution ultrasound your breast cancer treatment will carry on as normal. This means that when the research study stops, your ongoing treatment and care will not be affected.

11. What if something goes wrong?

Maidstone and Tunbridge Wells NHS Trust holds standard NHS Hospital Indemnity and insurance cover with NHS resolution for NHS Trusts in England, which apply to this study. This does not affect your legal rights to seek compensation. If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the research team. The normal National Health Service complaints mechanisms are also available to you.

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be fully addressed. If you have a concern about any aspect of this study, you should ask to speak with the doctors or chief investigator (Miss Karina Cox, karina.cox@nhs.net), who will do their best to answer your questions. If you are still unhappy and wish to complain formally, you can contact the Patient Advisory Liaison Service (PALS):

Telephone: 01892 638237/632953

By email: mtw-tr.palsoffice@nhs.net

12. How will we use information about you?

Maidstone and Tunbridge Wells NHS Trust are the sponsors for this study. Maidstone and Tunbridge Wells NHS Trust will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Maidstone and Tunbridge Wells NHS Trust will keep your personal data for:

10 years after the study has finished in relation to data subject consent forms.

10 years after the study has completed in relation to primary research data.

Certain members of the research team will need to use information from your medical records for this research project. This information will include your initials, NHS number, name, date of birth (which will be used to calculate age), and contact details. Other members of the research team who do not need to know these details will not be able to see your name or contact details and your data will have a specific code number instead.

We will keep all information about you safe and secure.

Once we have finished the study, we will keep some of the data so we can check the results. We will write our reports in a way that keeps your identity secure and prevents others from knowing that you took part in the reasearch.

Legal basis

As an NHS Hospital Trust, we use personally-identifiable information to conduct research to improve health, care and services. As a publicly-funded organisation, we have to ensure that it is in the public interest when we use personally-identifiable information from people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use your data in the ways needed to conduct and analyse the research study.

Health and care research should serve the public interest, which means that we have to demonstrate that our research serves the interests of society as a whole. We do this by following the UK Policy Framework for Health and Social Care Research.

International transfers

There may be a requirement to transfer information to countries outside the UK and European Economic Area (for example, to a research partner). Where this information contains your personal data, Maidstone & Tunbridge Wells NHS Trust will ensure that it is transferred in accordance with data protection legislation. If the data is transferred to a country which is not subject to a European Commission (**EC**) adequacy decision in respect of its data protection standards, Maidstone & Tunbridge Wells NHS Trust will enter into a data sharing agreement with the recipient organisation that incorporates UK/ EC approved standard contractual clauses that safeguard how your personal data is processed.

Sharing your information with others

For the purposes referred to in this privacy notice and relying on the bases for processing as set out above, we will share your personal data with certain third parties.

Other NHS Trust employees, agents, contractors and service providers (for example, suppliers of printing and mailing services, email communication services or web services, or suppliers who help us carry out any of the activities described above). During the study, all hard copy documents containing participants identifiable data (e.g. consent forms) will be stored in locked filing cabinets within alarmed, access restricted hospital buildings. Only the study team will have access to these locked cabinets. Electronic data will only be accessible via a password protected database held on a secure server. Our third party service providers are required to enter into data processing agreements with us. We only permit them to process your personal data for specified purposes and in accordance with our policies.

What are your choices about how your information is used?

You can stop being part of the study at any time, without giving a reason, but we will keep information about you that we already have. We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

Where can you find out more about how your information is used ?

You can find out more about how we use your information

at www.hra.nhs.uk/information-about-patients/

by asking one of the research team

by sending an email to Miss Karina Cox (karina.cox@nhs.net).

Complaint

If you wish to raise a complaint on how we have handled your personal data, you can contact Maidstone and Tunbridge Wells NHS Trust Data Protection Officer (XXXXXX) who will investigate the matter. If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO) by visiting **https://ico.org.uk/make-a-complaint/** or by calling their helpline on 0303 1231113.

13. What will happen to the results of the research study?

The results of the study will be presented at bio-medical engineering and medical meetings and be published in bio-medical/ medical journals. You will also be sent a report of the study (with a link to the medical journal) using words that can be easily understood by people who do not have a background in medicine or science. You will not be identified in any report/ publication.

14. Who is organising and funding the research?

The research is being funded by Breast Cancer Kent and Maidstone & Tunbridge Wells NHS Trust. A research team composed of biomedical engineers at Imperial College London, breast cancer doctors at Maidstone and Tunbridge Wells NHS Trust and Cancer biologists at The University of Kent have organized the research.

The doctor conducting the research is not being paid to include you in the study.

15. Who has reviewed the study?

The West os Scotland Research Ethics Committee have reviewed this study and have given ethical approval.

Thank you for taking time to read this and for considering taking part in this study.

6.3 Ethical approval

WoSRES
West of Scotland Research Ethics Service

NHS Greater Glasgow and Clyde

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Paisley PA2 7DE
www.nhsggc.org.uk
Date 18 May 2022
Direct line 0141-314-0212
e-mail WosRec1@ggc.scot.nhs.uk

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

Dear Miss Cox

Study title:	**Imaging of tumour microvasculature using high resolution contrast enhanced ultrasound (CEUS) together with markers of proliferation/ angiogenesis/ vascular mimicry to characterise response to NACT in triple negative breast cancer.**
REC reference:	**22/WS/0045**
Protocol number:	**KC_09012022**
IRAS project ID:	**301345**

Thank you for your letter, responding to the Research Ethics Committee's (REC) request for further information on the above research and submitting revised documentation.

The further information was considered in correspondence by a Sub-Committee of the REC. A list of the Sub-Committee members is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Good practice principles and responsibilities

The UK Policy Framework for Health and Social Care Research sets out principles of good practice in the management and conduct of health and social care research. It also outlines the responsibilities of individuals and organisations, including those related to the four elements of research transparency:

1. registering research studies
2. reporting results
3. informing participants
4. sharing study data and tissue

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Number	Condition
1	In the GP letter, please add "There is no change to standard therapy and the only interventions are extra imaging and biopsies".

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Confirmation of Capacity and Capability (in England, Northern Ireland and Wales) or NHS management permission (in Scotland) should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA and HCRW Approval (England and Wales)/ NHS permission for research is available in the Integrated Research Application System.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All research should be registered in a publicly accessible database and we expect all researchers, research sponsors and others to meet this fundamental best practice standard.

It is a condition of the REC favourable opinion that **all clinical trials are registered** on a publicly accessible database within six weeks of recruiting the first research participant. For this purpose, 'clinical trials' are defined as:

- clinical trial of an investigational medicinal product
- clinical investigation or other study of a medical device
- combined trial of an investigational medicinal product and an investigational medical device

- other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice.

Failure to register a clinical trial is a breach of these approval conditions, unless a deferral has been agreed by the HRA (for more information on registration and requesting a deferral see: Research registration and research project identifiers).

If you have not already included registration details in your IRAS application form you should notify the REC of the registration details as soon as possible.

Publication of Your Research Summary

We will publish your research summary for the above study on the research summaries section of our website, together with your contact details, no earlier than three months from the date of this favourable opinion letter.

Should you wish to provide a substitute contact point, make a request to defer, or require further information, please visit:
https://www.hra.nhs.uk/planning-and-improving-research/application-summaries/research-summaries/

N.B. If your study is related to COVID-19 we will aim to publish your research summary within 3 days rather than three months.

During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you haven't already done so, please register your study on a public registry as soon as possible and provide the REC with the registration detail, which will be posted alongside other information relating to your project. We are also asking sponsors not to request deferral of publication of research summary for any projects relating to COVID-19. In addition, to facilitate finding and extracting studies related to COVID-19 from public databases, please enter the WHO official acronym for the coronavirus disease (COVID-19) in the full title of your study. Approved COVID-19 studies can be found at:
https://www.hra.nhs.uk/covid-19-research/approved-covid-19-research/

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

After ethical review: Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study, including early termination of the study
- Final report
- Reporting results

The latest guidance on these topics can be found at https://www.hra.nhs.uk/approvals-amendments/managing-your-approval/.

Ethical review of research sites

NHS/HSC sites

The favourable opinion applies to all NHS/HSC sites taking part in the study, subject to confirmation of Capacity and Capability (in England, Northern Ireland and Wales) or management permission (in Scotland) being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS/HSC sites

I am pleased to confirm that the favourable opinion applies to any non-NHS/HSC sites listed in the application, subject to site management permission being obtained prior to the start of the study at the site.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
GP/consultant information sheets or letters [GP Letter]	1.0	26 April 2022
IRAS Application Form [IRAS_Form_15032022]		15 March 2022
Letter from funder [Letter from Funder]		17 August 2021
Letter from sponsor [Sponsor letter]	1.0	14 February 2022
Non-validated questionnaire [TNBC_CEUS_Satisfaction Questionnaire]	2.0	26 April 2022
Non-validated questionnaire [Tracked Questionnaire]	2.0	26 April 2022
Other [Tracked Protocol]	3.0	26 April 2022
Participant consent form [TNBC_CEUS_Consent]	2.0	26 April 2022
Participant consent form [Tracked Consent]	2.0	26 April 2022
Participant information sheet (PIS) [TNBC_CEUS_PIS]	2.0	26 April 2022
Participant information sheet (PIS) [Tracked PIS]	2.0	26 April 2022
Referee's report or other scientific critique report [Breast Cancer Now Reviewer Feedback]	1.0	06 July 2021
Research protocol or project proposal [TNBC_CEUS_Protocol]	3.0	26 April 2022
Response to Request for Further Information [Response to REC]		
Summary CV for Chief Investigator (CI) [CI CV - Karina Cox]		05 April 2021
Summary CV for student [Student CV - Mr Jaideep Rait]	1.0	09 January 2022
Summary CV for supervisor (student research) [Supervisor CV - Mengxing Tang]		09 January 2022
Summary CV for supervisor (student research) [Supervisor CV - Karina Cox]	1.0	05 April 2021
Summary CV for supervisor (student research) [Supervisor CV - Catherine Harper-Wynne]	1.0	14 February 2022

Document	*Version*	*Date*
Summary CV for supervisor (student research) [Supervisor CV - Sonia Saw]	1.0	23 February 2022
Summary CV for supervisor (student research) [Supervisor CV - Michelle Garrett]	1.0	02 February 2022

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

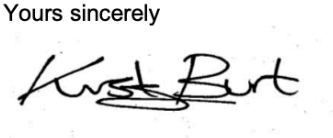
HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at:
https://www.hra.nhs.uk/planning-and-improving-research/learning/

IRAS project ID: 301345 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely



On behalf of
Dr Katriona Brooksbank
Chair

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments
"After ethical review – guidance for researchers"

6.4 GP information leaflet

GP Notification Letter

Miss Karina Cox

Consultant Breast and Oncoplastic Surgeon

Peggy Wood Breast Care Centre

Maidstone Hospital

Hermitage Lane

Maidstone

ME16 9QQ

GP practice

RE: Patient Name

Dear Dr.

Your patient has agreed to take part in the clinical trial detailed below.

Imaging of tumour microvasculature using high resolution contrast enhanced ultrasound (CEUS) together with markers of proliferation/ angiogenesis/ vascular mimicry to characterise response to neoadjuvant chemotherapy in triple negative breast cancer.

Your patient has been provided with an information sheet for the trial which explains why they have been approached to take part and that their participation is entirely voluntary. They are free to withdraw at any point without prejudicing their current or future medical care.

There is no change to standard therapy and the only interventions are extra imaging and biopsies. No change in management is required by you as their primary medical carer.

Should, you have any questions/ require further information about this research, please do not hesitate to contact me at karina.cox@nhs.net.

Yours sincerely,

Miss Karina Cox

(Chief Investigator)

6.5 SOP Tissue destruction

SOP Tissue Usage

PROJECT TITLE

Imaging of tumour microvasculature using high resolution contrast enhanced ultrasound (CEUS) together with markers of proliferation/ angiogenesis/ vascular mimicry to characterise response to NACT in triple negative breast cancer.

REC NO: 22/WS/0045

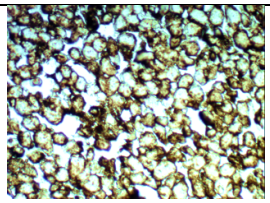
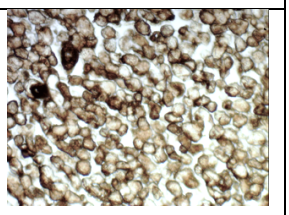
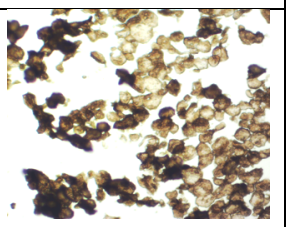
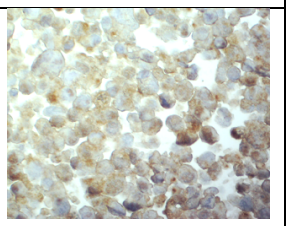
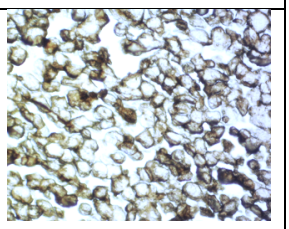
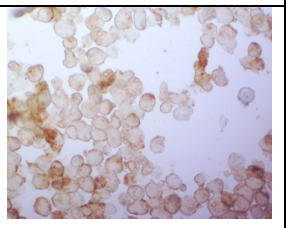
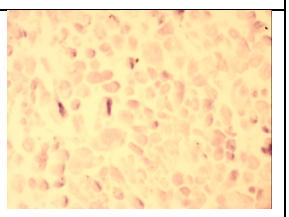
REC Authority: West of Scotland REC 1

IRAS ID: 301345SOP for tissue usage

As project is approved by recognised REC no HTA licence is required as per HTA guidance 2017 code E (Research) paragraph 84. Consent will have been agreed with the participants and the following will need to be adhered to ensure compliance with the Human Tissue Act (HTA 2004) and appropriate usage of human tissue.

1) Tissue of participants will be collected for study by Mr Jaideep Rait (MRes student and study co-ordinator) and delivered by himself to the histopathology laboratory at Maidstone Hospital for slide preparation.
2) Where possible slides for research within the study will be taken the same day, by himself to the Biosciences department at the University of Kent under the care of Professor Michelle Garrett and stored in a study-designated area.
3) If there is a delay in transporting then they will be stored in clearly marked bags identifying them as in transit to the University of Kent with the study name etc.
4) Samples transported will be anonymised to protect the confidentiality of study participants.
5) On completion of research on samples, they will be destroyed at the Biosciences laboratory as soon as feasible with all samples used in the project destroyed at the end of the full study. It is essential that no tissue is stored beyond the end of the study in compliance with the HTA Code E above.

6.6 HCC38 Positive Controls

Antibody, dilutions and magnification	HCC38
Ki67 200x 1:100	
EGFR 200x 1:100	
VEGFR 200x 1:100	
VEGFA 200x 1:100	
CD34 200x 1:100	
CD31 200x 1:200	
No Primary Antibody (negative control) 200x	

HCC38 - Immunohistochemistry with primary antibody (positive control) and no primary antibody (negative control)*All visualised as per materials and methods.*