FRONTIER Data Index Document

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SUMMARY

This document summarises the data available for secondary analysis that was accumulated from the FRONTIER trial. An open-labelled study to characterise Fluciclovine (18F) uptake measured by positRon emissiON Tomography In breast cancER

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# Study Overview

18F-fluciclovine is a synthetic amino acid positron emission tomography (PET) radiotracer that is approved for use in prostate cancer. In this clinical study, we characterised the kinetic model best describing the uptake of 18F-fluciclovine in breast cancer and assessed differences in tracer kinetics and static parameters for different breast cancer receptor subtypes and tumour grades.

This pre-operative observational imaging study will characterise the uptake of 18F-fluciclovine in different breast cancer subtypes and correlate uptake with gene expression and metabolomic profile. Fluciclovine is already used to identify recurrent prostate cancer, and one of the objectives of FRONTIER is to see if Fluciclovine works in breast cancers as it does in the prostate. Additionally, the study attempts to understand if genetic switches are turned on in the tumour which would allow to use this imaging technique to pick out the right treatment for patients.

# 2 Study Design

The study group includes 39 participants recruited from the Churchill Hospital in Oxford. Participants are post-menopausal women with histologically proven breast cancer measuring ≥ 1.5 cm scheduled to be treated by primary surgical resection. All participants underwent 20-min dynamic fluciclovine (18F) PET/CT of the breast tumour before surgery, Uptake into primary breast tumours was evaluated using one- and two-tissue reversible compartmental kinetic models and static parameters. Breast tumour samples were collected during surgery. Fluciclovine (18F) uptake by the tumour will then be compared to the tumour receptor subtype and results of the tumour sample analyses.

Clinical data includes MRI scans, NGS bams - mRNA, metabolomics, IHC.

## Inclusion Criteria

1. Biopsy-proven breast cancer (invasive ductal or ductal carcinoma in situ (DCIS) measuring ≥ 1.5cm.
	1. Tumour size should be based on the longest diameter measured on ultrasound, mammogram or MRI performed within 2 months before enrolment.
2. No prior treatment for breast cancer.
3. Female, Age ≥ 40 years.
4. The patient is willing and able to comply with the protocol scheduled visits and examinations for the duration of the study. Women of childbearing potential must follow contraception guidance given as a standard of care at breast cancer diagnosis.
5. Written (signed and dated) informed consent

# Trial Outcomes

A reversible one-tissue compartment model was shown to best describe tracer uptake in breast cancer. No significant differences were seen in kinetic or static parameters for different tumour receptor subtypes or grades. Kinetic and static parameters showed a good correlation.

18F-fluciclovine has potential in the imaging of primary breast cancer, but kinetic analysis may not have additional value over static measures of tracer uptake.

# Data Available for Use

## mRNA

36 paired samples of stroma and tumour from trial participants

FastQC was first utilised to identify adaptor sequences and remove low quality reads. Cutadapt was used to remove the universal Illumina adaptor sequences and a string of PolyAs, which was found to improve mapping efficacy for 3’ enriched sequencing. The FastQC step was repeated to ensure the adaptor sequences were appropriately removed. STAR was utilised to align the reads to the human genome with default settings before quantifying the aligned reads for each gene using featureCounts. Given 3’ sequencing is prone to multimapping, read quantification with featureCounts was trialled with or without the inclusion of multi-mapped reads as fractional counts.

The RNA-sequence-generated count matrix was processed using the DESeq2 pipeline for downstream and differential expression analysis. The matrix was filtered for low-count genes with less than ten counts in fifteen or more samples to bring the total number of genes from 58,735 to 21,800. Counts underwent variance stabilisation transformation using the vst function to remove the mean-variance correlation apparent in RNA-sequence data

## Metabolomics Data

The metabolomics data consists of raw and normalised quantity measurements for the 101 metabolites across 73 paired samples (tumour vs stroma).

Breast cancer tissue was subjected to mechanical disruption via the IKA Ultra-Turrax T-8 homogenizer. Subsequently, a hydrophilic extraction was carried out using a solution composed of methanol, acetonitrile, and water in a proportion of 50:30:20, with 250μL of the solution allocated for every 10 mg of homogenized tissue. After mixing, the samples were centrifugated at 10,000G, and supernatant stored at -80

For the LC separation, column A the sequant Zic-pHilic (150 mm × 2.1 mm i.d. 5 μm) with the guard column (20 mm × 2.1 mm i.d. 5 μm) from HiChrom, Reading, UK. Mobile phase A: 20 mM ammonium carbonate plus 0.1% ammonia hydroxide in water. Mobile phase B: acetonitrile. The flow rate was maintained at a constant rate of 180 μL/minute, while the gradient was set as follows: from 0 to 1 minutes, 70% of solvent B was used; from 1 to 16 minutes, 38% of solvent B was utilized; from 16.5 to 25 minutes, 70% of solvent B was employed. The Thermo Q-Exactive Orbitrap mass spectrometer was employed in a polarity-switching fashion. Researchers conducting analyses on metabolomics samples were deliberately kept unaware of the experimental interventions, and sample randomization was implemented to prevent any potential discrepancies caused by machine drifts.

Metabolomic normalisation, differential abundance, integrated enrichment and network analysis were performed using MetaboAnalystR. Metabolite abundances were normalised by dividing each by total library size, mean-centred and converted to log-scale. For visualisation, PCA and global differential abundance, missing values were replaced by 1/5th of the minimum positive values of their corresponding variable. For additional analysis, minimum values were contained as NAs.

## PET/CT imaging

Patients were imaged supine with their arms by their side using a Discovery 710 PET/CT scanner (GE Healthcare). They were injected with 370 MBq (±10%) of 18F-fluciclovine 30 s into the 20 min dynamic list-mode PET acquisition, which was centred over the breasts. Before each PET acquisition, a CT scan was performed for localisation and PET attenuation correction. PET data were reconstructed using a time-of-flight ordered subset expectation maximisation algorithm (VPFX, GE Healthcare) with a standard 6.4 mm Gaussian filter applied post-reconstruction. 18F-Fluciclovine uptake in tumours was measured for each 5-min time interval. SUVmax and SUVpeak were calculated using Hermes Hybrid Viewer (Hermes Medical Solutions AB) for each interval, to determine which period demonstrated the highest level of uptake.

CT format: file\_name.nii.gz

## IHC Data

## This data contains the IHC scores for 5 proteins (ASCT2, LAT1, pS6, ki67, SNAT2) split across two independent pathologists (CS) and (JL) which are affixed to each protein descriptor.

ASCT2, LAT1, pS6 and SNAT2 were classified 1 to 3 and ki67 by percentage.

H&E images format:   file\_name.qptiff

IF images format: file\_name.qptiff

# Participant Information

|  |  |  |
| --- | --- | --- |
| Category | Characteristic | Number of patients |
| Tumour histology | IDC | 39 |
| Tumour Grade | 1  | 7 |
|  | 2  | 12 |
|  | 3  | 20 |
| Receptor subtype\* | ER+ HER2 | 24 |
|  | HER2+  | 8 |
|  | TN | 7 |
| On metformin | No | 35 |
|  | Yes | 4 |
| Metformin dose |  |  |
| Tumour diameter (mm) | Median (Range) | 24 (15-70) |
| Method label (used to determine tumour diameter) | Mammogram/MRI/ultrasound |  |
| Neoadjuvant chemotherapy | Received before surgery (y/n) |  |

### Table 1: Participant information

\*Oestrogen receptor Allred score of 2/8 or less was considered negative. For HER2 a score of 3+ on immunohistochemistry was considered positive, and if scored borderline (2+), HER2 in situ hybridisation testing was used as per current UK guidance. Receptor status was determined using immunohistochemistry by the Cellular Pathology Laboratory at the John Radcliffe Hospital.

# Data QC

FastQC was first utilised to identify adaptor sequences and remove low quality reads.

Exploratory analysis first assessed whether the datasets captured the hallmarks of breast cancer pathology. Principal component analysis (PCA) for normalised metabolite abundance and gene mRNA measures separated most stroma and tumour samples. Accordingly, differential expression and abundance analyses identified many altered genes and metabolites indicative of cancer's global transcriptional and metabolic reprogramming. The top differentially expressed genes included known prognostic markers for breast cancer, such as SIX4, KIF4A, and ASPM. Additionally, metabolomics suggests increased glucose usage and lactate production in the tumour samples, as expected with the Warburg effect. At the pathway level, enrichment analysis identified several terms related to proliferation and oncogenic signalling, including MYC and mTOR. In agreement with the enrichment for oestrogen pathways, clustering of tumour samples via oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) expression established three distinct clusters, which relate to the main subtypes of breast cancer: ER+, HER2+, and triple-negative. Finally, targeted DNA sequencing identified breast cancer-related mutations, such as KMT2C, one of the most frequently mutated genes in ER+ patients. Therefore, this study has likely captured the genetic and metabolic changes dictating breast cancer development.

# References

Scott, N.P., Teoh, E.J., Flight, H. *et al.* Characterising 18F-fluciclovine uptake in breast cancer through the use of dynamic PET/CT imaging. *Br J Cancer* **126**, 598–605 (2022). https://doi.org/10.1038/s41416-021-01623-3