METFORMIN Data Index Document

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**Summary**

This document summarises the data available for secondary analysis that was accumulated from the METFORMIN trial (Effect of Metformin on Breas Cancer metabolism, NCT01266486). Thirty-six patients with untreated primary breast cancer were recruited to a window study and transcriptomic profiling of tumour samples carried out before and after metformin treatment.

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# Study Overview

# Epidemiological and retrospective clinical studies suggest a reduction in the relative risk of cancer associated with the diabetes drug, metformin. Multiple phase 3 clinical trials are now underway to assess the potential of repurposing metformin as an anti-cancer therapy. However, metformin’s anti-cancer mechanism of action remains unclear. The canonical view is that metformin activates 5′ AMP-activated protein kinase (AMPK) in cancer cells leading to metabolic reprogramming and inducing a limit on utilisation of nutrient resources, subsequently halting proliferation. The suggestion is that metformin modulates mitochondrial metabolism at clinical doses.

# In this clinical pharmacodynamic study of 36 breast cancer patients, we show that metformin treatment reduces the levels of mitochondrial metabolites, activates multiple mitochondrial metabolic pathways, and increases 18-FDG flux in tumours. Two tumour groups are identified with distinct metabolic responses, an [OXPHOS](https://www.sciencedirect.com/topics/medicine-and-dentistry/oxidative-phosphorylation) transcriptional response group for which there is an increase in [OXPHOS](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/oxidative-phosphorylation) [gene transcription](https://www.sciencedirect.com/topics/medicine-and-dentistry/genetic-transcription) and an FDG response group with increased 18-FDG uptake.

# Furthermore, multiple genes regulating fatty acid oxidation are upregulated at the transcriptomic level following metformin treatment. Additionally, an increase in expression of a composite mitochondrial FAO gene expression profile correlated with changes in a proliferation gene signature. This proliferation signature discriminates between patient groups with different metabolic responses.

# Study Design

Patients were recruited from the [medical oncology](https://www.sciencedirect.com/topics/medicine-and-dentistry/medical-oncology) breast cancer clinic over a period of 30 months between May 2011 and November 2013 in three UK centres, Oxford, Luton and Dundee. All patients at the point of recruitment had been referred with a view to [neoadjuvant chemotherapy](https://www.sciencedirect.com/topics/medicine-and-dentistry/neoadjuvant-chemotherapy) and had histologically confirmed breast cancer. In all cases the primary breast cancer was *in situ* and no patients had received any prior treatment for breast cancer. In total 41 patients were recruited and had evaluable data.

Metformin was given in the Glucophage XR formulation in an escalating dose once daily for a minimum of 13 days and a maximum of 21 days (500mg for days 1**–**3, 1000mg for days 4**–**6 and 1500mg thereafter). The day before commencing metformin a core biopsy was taken under ultrasound guidance from the periphery of the primary tumour. Within 1 minute of this procedure, the biopsy material was snap-frozen in [liquid nitrogen](https://www.sciencedirect.com/topics/medicine-and-dentistry/liquid-nitrogen) before storage at -80°C. Prior to metabolomics analysis [biopsy samples](https://www.sciencedirect.com/topics/medicine-and-dentistry/biopsy-sample) were divided and one portion was used for broad metabolomics analysis and the other to generate a [lipid profile](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/lipid-profile)

2.1 Inclusion criteria

|  |  |
| --- | --- |
| Inclusion criteria | Exclusion criteria |
| Women with a histology-proven in situ primary breast cancer ≥2 cm in diameter | Radiotherapy, major surgery, significant traumatic injury, endocrine therapy, immunotherapy, chemotherapy or experimental therapy during four weeks prior to starting or during the trial |
| Eastern Cooperative Oncology Group (ECOG) performance status 0–1 | Pregnancy or breastfeeding |
| Age >18 years | History of type 1 or type 2 diabetes |
| Fasting or random serum glucose less than 7.0 mmol/L | Treatment with metformin in the past year |
| No prior treatment for breast cancer and scheduled to commence neoadjuvant chemotherapy in 3 weeks’ time | Estimated glomerular filtration rate |
| Have given written informed consent and are capable of cooperating with protocol | Acute or chronic metabolic acidosis |
| Adequate bone marrow, renal and liver function | Known hypersensitivity to metformin |

Table 1: Inclusion and exclusion criteria

# Data

3.1 Samples available

|  |  |  |
| --- | --- | --- |
|  |  | N of Patients |
| Recruitment and samples analysed | Total patient recruitment to study | 41 |
| Number of paired PET-CT scans available for analysis | 36 |
| Number of paired tumour samples with sufficient material for analysis | Metabolomics | 29 |
| RNASeq | 36 |

Table 2: Samples available

3.2 Tumours and patients characteristics

|  |  |  |
| --- | --- | --- |
| ER/HER2 status | ER positive | 32 |
| ER negative | 9 |
| HER2 positive | 8 |
| HER2 negative | 33 |
| Triple negative (ER negative and HER2 negative) | 8 |
| Tumour type | Ductal carcinoma | 32 |
| Lobular carcinoma | 7 |
| Mixed ductal and lobular carcinoma | 1=Mixed Ductal & Lobular1=Main Mass unusual morphology |
| Grade 1 | 2 |
| Grade 2 | 24 |
| Grade 3 | 15 |
| Median tumour size (on magnetic resonance imaging) | 49mm (range 30–147) |
| Patient characteristics | Median age at study entry | 49 years (range 27–67) |
| Median body mass index | 28.1 (range 19.6–45.3 |
| Height |
| Weight |
| Patient characteristics Pre- and post-intervention | Glucose Total cholesterol, HDL cholesterol, Triglyceride, Lactate, Insulin, Insulin.1, C peptide, IGF1, IGF2, IGF BP3, Leptin, HOMA, p4EBP1 in tumour nucleus, p4EBP1 in tumour cytoplasm, P56RP in tumour cytoplasm, pAMPK in tumour nucleus, S Adenosyl Methionine, Glycerylphosphorylethanolamine, Aspartate, N AcetylAspartate, Myristic Acid, GlucoseNa, NAD, Urea, P56RP, Linoleic Acid, Palmitoleic Acid, Palmitic Acid, GSH, Creatinine, Oleic Acid, Propionylcarnitine, Stearic Acid, Arginine, Glycerylphosphorylcholine, Pyruvate, MRI Breasts Baseline Tumour size, X3 Hydroxybutyrate, Fumarate, Malate, S Adenosyl Homocysteine, Adenine, Acetylcarnitine, Succinate, AMP, Alanine, Asparagine, Glutamate, Hexose, Tryptophan, pAMPK tumour nuclear, IGF1 IGF2, ADP, HexosePhosphate, Glycerate, Pyroglutamic Acid, ATP, Gluconate, Ornithine, IGF1, Serine, Insulin.1, Insulin, Triglyceride, BMI, Proline, ButyrylCarnitine, IGF1 IGF BP3 ratio, Glutamine, Adenosine, Leucine, AcetylCholine, Citrate, Age Study entry, Xylulose5Phosphate, Hypoxanthine, Ribulose, Sample TimePoint, Dihydrothymine, Valine, IGF BP3, Phenylalanine, MRI Breasts Radiological response, Leptin, Lactate, Glycine, Threonine, Choline, Ribose Ribulose 5Phosphate, Methionine, GSSG, Ethanolamine, pCR RATE 1 . pCR, Taurin, Uracil, Guanine, HOMA, Homocysteine, Cytosine, Lactate 1, Sedoheptulose 7 phosphate, Kynurenine, Ki67 nuclei stained, MRI Breasts Comments, pAMPK tumour cytoplasm, Carnitine, Nicotinamide, Histidine, C peptide, IGF2, Citrulline, Tyrosine, MRI Breasts Kuhl curve type, Lysine  |

Table 3: Tumours, patients and samples characteristics recorded

3.3 RNASeq

Samples are pre-metformin administration breast cancer biopsies from the periphery of the primary tumour.

Next-generation sequencing of ‘Poly (A) targeted’ mRNA was carried out for the clinical biopsy samples taken pre- and post-metformin. The fold change of normalised expression level, FPKM (Fragments Per Kilobase of transcript per Million mapped reads), for each gene, was then estimated from those aligned reads using Cuffdiff 2.2.1. Non-parametric rank product (R project v3.3.1) was used to prioritise the genes with a statistically significant change in abundance (FDR < 0.05) between pre- and post-metformin treatment. Methods used to estimate significance included one-way ANOVA and unpaired Student’s *t*-test

3.4 Immunohistochemistry

Staining for p-AMPK (1 in 200, thr172 residue, [Cell Signaling](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/signal-transduction) Technologies #2535) and pAKT (1 in 100, ser473 residue, [Cell Signaling](https://www.sciencedirect.com/topics/medicine-and-dentistry/signal-transduction) Technologies #4060), was performed on a Leica Bond-max autostainer.

Quantitative scoring of the staining of complete tumour sections was evaluated by two accredited [pathologists](https://www.sciencedirect.com/topics/medicine-and-dentistry/pathologist) using high-power fields the intensity of the [immunostaining](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/immunostaining) was classified into 4 categories: 0, no immunostaining present; 1, weak staining; 2, moderate staining; and 3, strong staining and the percentage of positive cells at each intensity was then classified into 4 groups; 1 (0-10% positive cells), 2 (11% to 50% positive cells), 3 (51% to 80% positive cells) or 4 (81 to 100% positive cells). The H-score of [immunoreactivity](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/immunoreactivity) was obtained by multiplying the intensity and percentage scores.

3.5 Pet-CT

The [radiotracer](https://www.sciencedirect.com/topics/medicine-and-dentistry/radioactive-tracer), 2-deoxy-2-(18F)fluoro-D-glucose (18[F]-FDG), was used for all examinations. Before scanning, patients were fasted overnight for at least 8 hours but could drink water. Patients’ [blood glucose](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/glucose-blood-level) was checked just prior to the scan with a portable [blood glucose](https://www.sciencedirect.com/topics/medicine-and-dentistry/blood-glucose) monitor to ensure it was <7mmol/L. All scans took place on either a 3D mode time of flight GE Discovery 690 64-slice PET-CT system (GE Healthcare, Milwaukee) or Siemens Biograph mCT-128 (Siemens Healthcare, Germany).

A dynamic acquisition of the breast tumour (and any lymph nodes within the PET field of view) was initiated with the patient imaged supine. Patients were injected with 18[F]-FDG (3 MBq/kg, up to a maximum of 400 MBq) 30 seconds into PET imaging, which continued for 45 minutes. The 45 minutes of data were then reconstructed as a sequence of images describing average activity concentrations during a series of time frames (1x30s, 12x5s, 6x10s, 5x30s, 10x60s, 6x300s).

50 minutes after injection, a static [PET scan](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/positron-emission-tomography) was performed from [skull base](https://www.sciencedirect.com/topics/medicine-and-dentistry/skull-base) to mid-thigh, acquiring data for four minutes at each bed [position](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/position). Thus the primary breast cancer was scanned at approximately 60 minutes post injection, in addition to the dynamic PET scanning from 0-45 minutes. Prior to each PET acquisition a CT scan was performed for localization and PET attenuation correction, using a pitch of 0.984, 120 kV, automA with a noise index of 25.

The PET images were reconstructed on a matrix of 5.5×5.5×3.3 mm3 voxels using filtered back projection for the dynamic sequence, and iterative reconstruction for the static scan. See [Supplemental Information](https://www.sciencedirect.com/science/article/pii/S1550413118305229?via%3Dihub" \l "app2) for further details of static and kinetic analysis of imaging.

# Data QC

Data quality has been checked using Rosner's generalized extreme Studentized deviate test as well as by graphical presentations.

# References

Lord SR, Cheng WC, Liu D, et al. Integrated Pharmacodynamic Analysis Identifies Two Metabolic Adaption Pathways to Metformin in Breast Cancer. Cell Metab. 2018;28(5)

Lord SR, Collins JM, Cheng WC, Haider S, Wigfield S, Gaude E, Fielding BA, Pinnick KE, Harjes U, Segaran A, Jha P, Hoefler G, Pollak MN, Thompson AM, Roy PG, English R, Adams RF, Frezza C, Buffa FM, Karpe F, Harris AL. Transcriptomic analysis of human primary breast cancer identifies fatty acid oxidation as a target for metformin. Br J Cancer. 2020 Jan;122(2):258-265.